



ABIOTIC AND BIOTIC STRESS IN PLANTS

THOMAS FLYNN

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Thomas Flynn

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Preface

Biological changes occurring in plants due to a change in external environmental conditions are referred to as stress. These external changes can trigger a wide array of plant responses such as altered gene expression, as well as changes in growth rates and crop yields. Plant stresses are classified into abiotic and biotic stress. Abiotic stresses cause major loss of crops worldwide. These include drought, floods, salinity and extreme temperatures. Shortage of water and increasing desertification are further increasing abiotic plant stress. Biotic stresses include infestation by nematodes, fungi, bacteria and herbivores. These stress causing agents deprive nutrients from the host plants and are responsible for major pre and post-harvest losses. To combat these stresses, plants have developed various mechanisms. This book unravels the recent studies in the field of abiotic and biotic stress in plants. It includes some of the vital pieces of work being conducted across the world, on various topics related to abiotic and biotic stresses in plants. This book will serve as a valuable source of reference for graduate and post graduate students.

The researches compiled throughout the book are authentic and of high quality, combining several disciplines and from very diverse regions from around the world. Drawing on the contributions of many researchers from diverse countries, the book's objective is to provide the readers with the latest achievements in the area of research. This book will surely be a source of knowledge to all interested and researching the field.

In the end, I would like to express my deep sense of gratitude to all the authors for meeting the set deadlines in completing and submitting their research chapters. I would also like to thank the publisher for the support offered to us throughout the course of the book. Finally, I extend my sincere thanks to my family for being a constant source of inspiration and encouragement.

Editor

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Abscisic Acid Signalling as a Target for Enhancing Drought Tolerance

Ley Moy Ng

Abstract

Abscisic acid (ABA) is a vital hormone that confers abiotic stress tolerance in plants. The identification of PYR/PYL/RCAR proteins as bona fide ABA receptors and the subsequent elucidation of the structural mechanisms of the core ABA signalling pathway in recent years has provided new and powerful insights in targeting ABA signalling to enhance abiotic stress tolerance in agriculture. This chapter reviews the components and molecular mechanisms of the core ABA signalling pathway, as revealed by X-ray crystallography studies, and how these knowledge led to preliminary efforts in novel biotechnological developments to improve stress tolerance in plants.

Keywords: Abscisic acid signalling, ABA receptors, PYL/PYL/RCAR, abiotic stress tolerance

1. Introduction

The persistence of drought and climate change continues to cause heavy crop losses worldwide and pose a threat to the global food security. To meet the demands of a booming global population, the World Bank estimates that food production must increase by at least 50% in the year 2050 [1]. Increasing agricultural productivity is one practical solution to the food crisis, as has been demonstrated by the Green Revolution, which was estimated to have saved more than one billion people from famine [2]. However, such intensive farming can leave harmful impacts on the environment, such as land degradation and freshwater depletion. Agriculture

is the largest consumer of global freshwater resources [3], and it is predicted that in the year 2030, the world will be in a 40% water shortage [4]. Therefore, in view of the water and food crises, solutions that aim to increase crop productivity while limiting agricultural water consumption are the most valuable. Such solutions may potentially arise from the fine understanding of how plants perceive and respond to abiotic stress signals.

The plant hormone abscisic acid (ABA) was first discovered in the 1960s and shortly after, its role as the central and critical regulator of abiotic stress response has become clear. Under environmental stress such as drought, ABA levels rise strongly and rapidly, triggering stress tolerant effects, such as stomatal closure, to enable the plants to conserve water and survive through the harsh condition [5]. Exogenous ABA treatment has been shown to maintain the survival and quality of plants subjected to drought stress [6]. However, the agricultural use of ABA has been limited by its short-lived bioactivity, which is due to its chemical instability and rapid catabolism in plants [7]. Therefore, there has been much interests in the development of synthetic compounds that functionally mimic ABA but exhibit longer periods of bioactivity. While earlier attempts of designing ABA analogues guided by the hormone's chemical structure and catabolic pathway had not led to any desirable candidates [8], recent approaches based on the molecular mechanisms of ABA receptor signalling has shown more promising results. Such developments have made been possible by advances in the structural mechanisms of ABA signalling components that have emerged in the past few years, as reviewed in the following sections.

2. ABA is a regulator of abiotic stress tolerance

Stress signals, such as drought, salinity, and temperature extremes, trigger the biosynthesis of ABA from carotenoid precursors [9]. ABA is synthesised in vascular tissues and transported to the roots and leaves where the actions occur [10]. In leaves, ABA induces stomata closure to prevent transpirational water loss and promotes the accumulation of osmocompatible solutes to retain water [11]. ABA inhibits root and shoot growth and promotes seed dormancy. Such inhibitory effects help plants to pull through adverse conditions and germinate only when the conditions are favourable for growth. ABA also confers tolerance to freezing through the induction of dehydration-tolerance genes [12].

The effects of ABA has immense agricultural and economic value. Massive amounts of crops are lost to drought every year and the situation may worsen as climate change persists. The ability to manipulate ABA responses offers an innovative solution to alleviate crop loss and sustain agricultural yield in the face of the inevitable climatic change. Moreover, the activation of ABA responses may enhance agricultural water use efficiency, addressing concerns of the global water scarcity issue. A sound understanding of ABA signalling is critical to the successful development of approaches to manipulate ABA responses.

3. Components of the core ABA signalling pathway

3.1. ABA receptors

"There are things known and there are things unknown, and in between are the doors of perception." — Aldous Huxley

ABA is a messenger that carries the abiotic stress signal. The first step for the plant cells to be aware of the stress is to perceive the stress signal. The perception and transmission of the signal is carried out by ABA receptors, which recognise the ABA molecule and convey the message to downstream effectors.

Although the importance of ABA and many aspects of its signalling has been established following the discovery of the hormone in the 1960s, the identity of ABA receptors has remained elusive for almost half a century. Early efforts to identify ABA receptors employ forward genetics screens of ABA-insensitive mutants, which have instead identified several mediators of ABA signalling downstream of the receptors, such as PP2Cs and transcription factors [13, 14]. The use of alternative approaches by virtue of ABA binding has led to a number of putative candidates (FCA, CHLH, GCR2, GTG1, and GTG2) but none has been further substantiated [15]. In retrospect, these approaches had been futile for two reasons. First, the true identity of ABA receptors had been masked by genetic redundancy, and had thus eluded identification by classical forward genetic screens. Second, the identification of ABA-binding proteins had not addressed the links of the putative candidates to well-established components of ABA signalling.

The discovery of the PYR/PYL/RCAR family of proteins as bona fide ABA receptors had been different from the earlier attempts. Several groups had independently discovered this family of novel candidates, which fit elegantly into a model that connected the core components of the ABA signal transduction pathway. The first landmark studies were two reports published in *Science* in May 2009 [16, 17]. To overcome genetic redundancy issues, Park et al. [17] used a selective ABA agonist, pyrabactin, in a chemical genetic screen of mutagenised *Arabidopsis* seeds and identified *PYR1* (for pyrabactin resistance 1). *PYR1* belongs to the START domain superfamily of proteins that includes the mammalian STARD proteins. By homology, 13 other *Arabidopsis* *PYR1*-like (PYL) members were identified and named PYL1–13 (Table 1). In a different approach, Ma et al. [16] screened for interactors with the type 2C protein phosphatase (PP2C) *ABI2*, which is a known component of ABA signalling, and identified the same 14 START domain family members, naming them as RCAR1–14 (Regulatory Component of ABA Response) (Table 1). For simplicity, the PYR/PYL/RCAR members are referred to in this chapter as PYL proteins. Other similar studies have also identified PYL members by their interactions with PP2Cs [18, 19] and collectively, these studies showed that PYL proteins are able to bind to ABA and in the presence of ABA, PYL proteins interact with and inhibit the ABA-signalling PP2Cs.

Members of the PYR/PYL/RCAR family

Protein Name			Solved Structures		
PYR/PYL	RCAR	Mutation(s)	Complex with		PDB ID
			Ligand	PP2C	
PYR1	RCAR11				3K3K
			ABA		3K3K, 3K90
			Pyrabactin		3NJO
		AS6			3WG8
			ABA	HAB1	3QN1
		H60P	ABA	HAB1	3ZVU
		K59R, V81I, F108A, F159L	Mandipropamid	HAB1	4WVO
PYL1	RCAR12				3KAY
			ABA		3JRS
			Pyrabactin		3NEF, 3NEG
			ABA	ABI1	3KDJ, 3JRQ
			Pyrabactin	ABI1	3NMN
					3KDH, 3KL1, 3KAZ
			ABA		3KB0, 3KDI
PYL2	RCAR14		Pyrabactin		3NJ0, 3NMH, 3NR4, 3NS2
		V114I	Pyrabactin		3NJ1
		A93F	Pyrabactin		3NMP
		A93F	Pyrabactin	HAB1	3NMT
		A93F	Pyrabactin	ABI2	3NMV
			ABA	HAB1	3KB3
			ABA	ABI2	3UJL
PYL3	RCAR13		Quinabactin (AM1)	HAB1	4LG5, 4LA7
			AM2	HAB1	4LGA
			AM3	HAB1	4LGB
					3KLX
			ABA		4DSB, 4DSC
			Pyrabactin		3OJI
			(-)-ABA		4JDA

		ABA	HAB1	4DS8
PYL4	RCAR10			
PYL5	RCAR8			4JDL
PYL6	RCAR9			
PYL7	RCAR2			
PYL8	RCAR3			
PYL9	RCAR1	ABA		3OQU, 3W9R
				3UQH, 3RT2
PYL10	RCAR4	ABA		3R6P
			HAB1	3RT0
PYL11	RCAR5			
PYL12	RCAR6			
PYL13	RCAR7		PP2CA	4N0G
Solved Structures				
Protein Name	Complex with		PDB ID	
	PYR/PYL/RCAR	SnRK2		
ABI1	PYL1-ABA			3KDJ, 3JRQ
	PYL1-Pyrabactin			3NMN
ABI2				3UJK
	PYL2-ABA			3UJL
	PYL2(A93F)-Pyrabactin			3NMV
	PYR1-ABA			3QN1
HAB1	PYR1(H60P)-ABA			3ZVU
	PYR1(K59R, V81I, F108A, F159L)-Mandipropamid			4WVO
	PYL2-ABA			3KB3
	PYL2(A93F)-Pyrabactin			3NMT
	PYL2-Quinabactin (AM1)			4LG5, 4LA7
	PYL2-AM2			4LGA
	PYL2-AM3			4LGB
	PYL3-ABA			4DS8
	PYL10			3RT0
		SnRK2.6		3UJG
PP2CA	PYL13			4N0G

Subset of Group A PP2CS

Subclass III SnRK2s

Protein Name	Solved Structures		
	Mutation(s)	Complex with	PDB ID
SnRK2.2			
SnRK2.3	D57A, K58A		3UC3
	D59A, E60A		3UC4
	D160A		3ZUT
	D160A, S175D		3ZUU
	S7A,S29A,S43A,C131A,C137A,C159A,S166A,T1 76A		3UDB
	HAB1		3UJG

Table 1. List of members belonging to the components of the core ABA signalling pathway and their solved structures.

3.2. Group 2C Protein Phosphatases (PP2Cs)

PP2Cs are a group of monomeric Mg²⁺/Mn²⁺-dependent serine/threonine phosphatases found in virtually all organisms and are known for their roles in the regulation of cell growth and cellular stress signalling. In *Arabidopsis*, there are 76 known PP2Cs that are genetically clustered into 10 groups (A-J), with the exception of 6 genes that could not be clustered [20]. At least 6 of the 9 members of group A PP2Cs have been shown to be involved in ABA signalling. Among these, ABI1, ABI2, and HAB1 are the most well-studied members and are known to be the negative regulators of ABA signalling.

The *abi1* and *abi2* (ABA insensitive) mutants were isolated from genetic screens of ABA insensitive mutants [21-24]. HAB1 was subsequently identified by homology to ABI1 and ABI2 [25]. The *abi1-1* (ABI1 G180D), *abi2-1* (ABI2 G168D), and *hab1* (HAB1 G246D) mutants displayed dominant ABA insensitive phenotypes, which are later known to be attributed to the loss of regulation by PYL proteins. The isolation of additional recessive loss-of-function mutations that resulted in ABA hypersensitive phenotype provided the early evidence that PP2Cs are negative regulators of ABA signalling [26-28]. This was further supported by the observations that double- or triple-PP2C-knockout mutants displayed enhanced ABA responses while constitutive expression of HAB1 led to reduced ABA sensitivity [28-30].

3.3. Snf1-related protein kinases 2 (SnRK2s)

While members of PP2Cs are known to play a negative regulatory role in ABA signalling, it is not surprising that a group of protein kinases are conversely the positive effectors. The *Arabidopsis* Snf1-related protein kinase (SnRK) group of kinases share a high degree of homology with the yeast Snf1 and catalytic subunits of mammalian AMPK. There are three classes of SnRKS in *Arabidopsis*, namely SnRK1, SnRK2, and SnRK3, which comprises of 3, 10, and 25 members, respectively [31]. SnRK2s are further divided into subclasses I, II, and III. While members of subclass I are not responsive to ABA, subclass II members are weakly

activated by ABA. All three members of subclass III, namely SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E/OST1, are strongly activated by ABA and are known to be the main positive regulators of ABA signalling. SnRK2.6 is known for its role in stomata regulation in guard cells [32, 33], while SnRK2.2 and SnRK2.3 are important in the inhibition of seed germination and root growth [34]. Consequently, triple mutants lacking SnRK2.2, SnRK2.3, and SnRK2.6 are deficient in almost all ABA responses, indicating that class III SnRK2s play a central role in the core ABA signalling pathway [35-37].

Active SnRK2s are autophosphorylated and are able to directly phosphorylate target proteins such as ion channels and transcription factors to elicit the ABA response. ABA-induced stomata closure is mediated by the effects of SnRK2.6 on ion channels. Under ABA stimulation, SnRK2.6 activates the slow-type anion channel SLAC1 and inhibits the inward-rectifying potassium channel, KAT1, by the phosphorylation of these ion channels, resulting in stomata closure [38-40].

ABA induction of target gene expression is mediated by the SnRK2 phosphorylation of transcription factors known as ABRE-binding (AREB) proteins or ABRE-binding factors (ABFs). The AREB/ABFs are basic domain leucine zipper (bZIP) transcription factors that recognises the ABA-responsive elements (ABREs) located in the promoters of ABA-responsive genes. Of the nine AREB/ABF homologs found in Arabidopsis, the AREB1/ABF2, AREB2/ABF4, and ABF3 were found to be master transcription factors responsible for regulating the ABRE-dependent expression of stress-responsive genes [41-44]. Such genes include Late Embryogenesis Abundant (LEA)-class genes, transcription factors, and mediators of ABA signalling.

4. Structural mechanisms of ABA receptor signalling

The core ABA receptor signalling cascade is comprised of the PYL ABA receptors, PP2Cs and SnRK2s (Figure 1). In the absence of ABA, PP2Cs such as ABI1, ABI2, and HAB1 inhibit the activities of SnRK2s, thus silencing the ABA response. During abiotic stress, ABA is generated rapidly and binds to the PYL proteins. ABA binding induces conformational changes to the PYL proteins, allowing the activated receptors to interact with and inhibit the PP2Cs. Consequently, the SnRK2s are relieved of inhibition by PP2Cs and are autoactivated by autophosphorylation. Active SnRK2s are able to phosphorylate their targets such as ion channels and AREB/ABF transcription factors to activate the ABA responses. Reconstitution of the core ABA signalling pathway has been demonstrated by co-expression of the core components (PYL, PP2C, SnRK2, and ABF) in plant protoplasts [45]. The following subsections will explain in detail the molecular mechanisms of each step of the core ABA signalling pathway.

4.1. Structure and properties of ABA receptors

To date, the structures of eight out of 14 PYL members have been solved either in their apo- or ligand-bound forms or in complexes with PP2C (Table 1). The overall PYL structures exhibit the helix-grip fold, a hallmark of START domain/Bet v 1-fold proteins, which is characterised

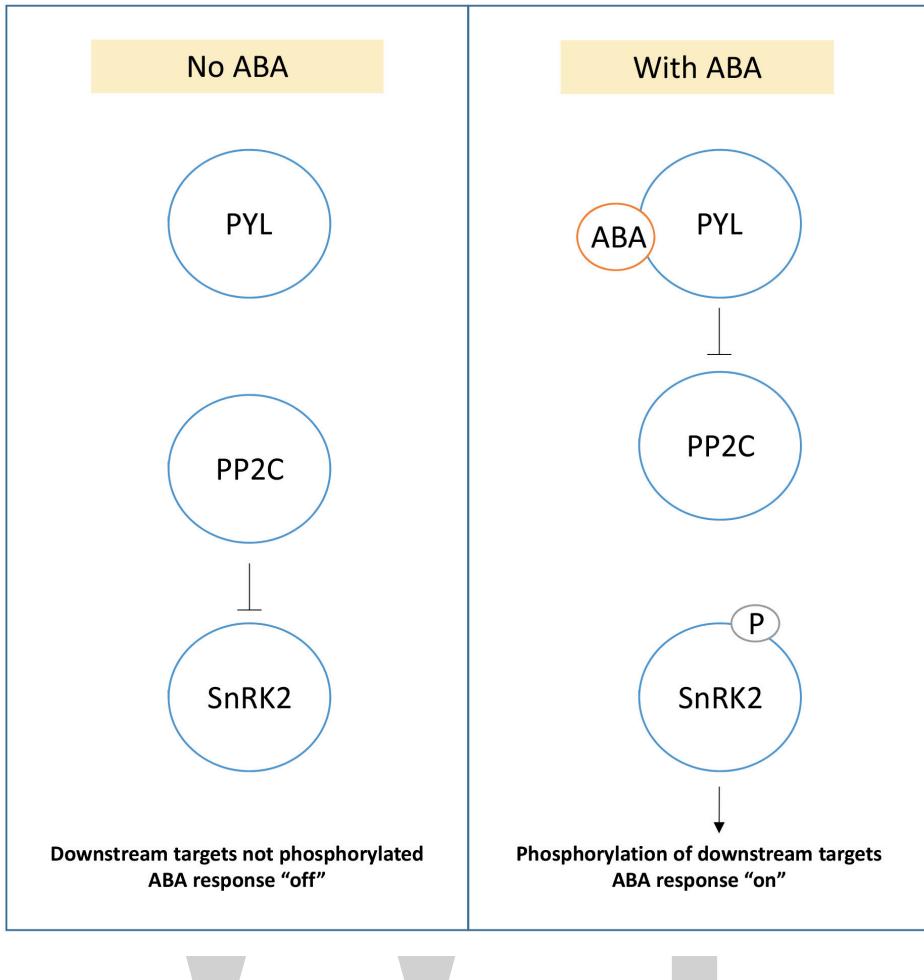


Figure 1. Summary model of the core ABA signalling pathway.

by the presence of a central β -sheet surrounded by N- and C-termini α -helices, with a long C-terminal α -helix packing tightly against the β -sheet (Figure 2). The helix-grip fold creates a large cavity constituting the ligand binding pocket.

Static light scattering and ultracentrifugation experiments showed that the apo PYR1, PYL1, and PYL2 are dimers in the solution, while PYL4–10, except for the untested PYL7, are monomers [46]. PYL3 exist in a monomer-dimer equilibrium. Consistently, the crystal structures of apo PYR1 and PYL1–3 revealed a cis-homodimer arrangement, with the two molecules associated in parallel orientation at their pocket entrance, thus hindering ligand entry [47–52]. Homodimeric receptors dissociate into monomers upon ABA binding. PYL3 has been shown to form a trans-homodimer intermediate that is able to bind ABA and dissociate into monomers more easily, consistent with its observed mixed monomer-dimer distribution [52]. While the dimeric receptors require ABA for their activity, the monomeric PYL members are able to inhibit PP2Cs constitutively in the absence of ABA, suggesting that receptor monomerisation is crucial for PP2C inhibition [46]. Despite the ABA-independent activity, it is important to note that the interactions of monomeric PYLs with PP2Cs are greatly enhanced

in the presence of ABA [16, 19, 46]. PYL13 is a divergent member that play a unique role in ABA signalling by its ability to heterodimerise with other PYL members and antagonise their activities [53]. While it was originally thought that PYL13 selectively inhibits PP2CA in an ABA independent manner [53, 54], recent evidence showed that PYL13 inhibits ABI1, ABI2, and PP2CA with the requirement of ABA [55].

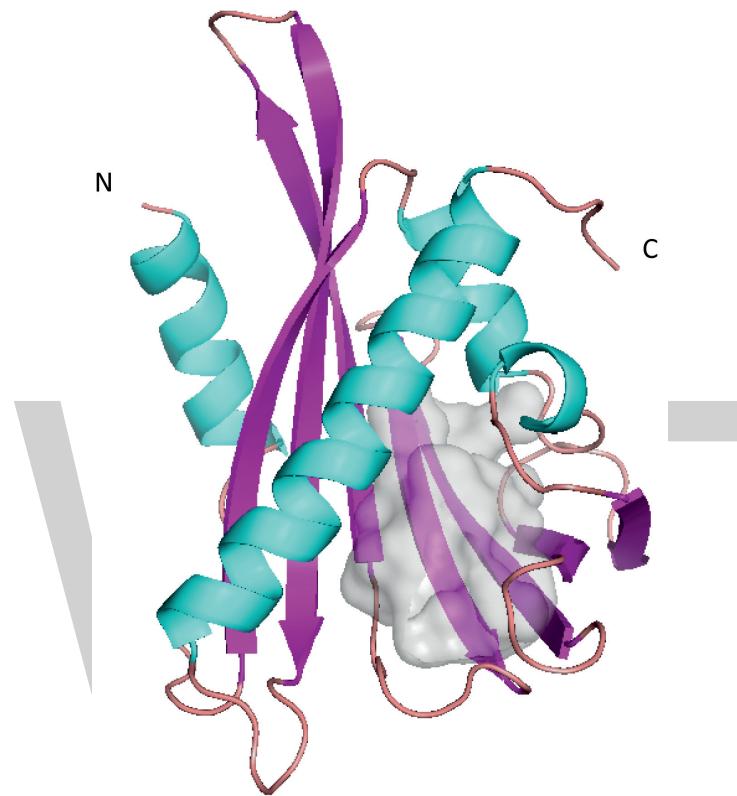


Figure 2. Structure of the ligand-free PYL2 ABA receptor (PDB code: 3KAZ) exhibiting the helix-grip fold. The ligand binding pocket is shown as surface presentation in grey.

4.2. ABA binding

The structures of PYL bound to ABA revealed a number of features in the ABA recognition. The entrance of the ligand pocket is surrounded by two functionally important β -loops that are known as the "gate" and "latch" loops [47] (alternatively named Pro-Cap and Leu-Lock [49], CL2, and CL3 [51], and the β 3– β 4 and β 5– β 6 lid loops [48, 50]), which contain the conserved amino acid sequences SGLPA and HRL, respectively. In the structure of the apo receptor, the gate loop appears to be in an open conformation to allow ligand access (Figure 3a). In the ABA-bound structure, the gate is in a closed conformation, making contact with the latch residues (Figure 3b).

Within the ligand pocket, ABA interacts with the receptor residues through a network of charged interactions, hydrogen bonds, and hydrophobic interactions [47-51]. ABA is anchored to the inner end of the ligand pocket by a direct charged interaction between its carboxylate group

and a conserved lysine residue (K59, K86, and K64 in PYR1, PYL1, and PYL2, respectively). Nearer to the outer end of the pocket, the cyclohexene ring of ABA interacts with the receptor gate and latch residues, thus pulling the gate loop into a closed conformation (Figure 6a).

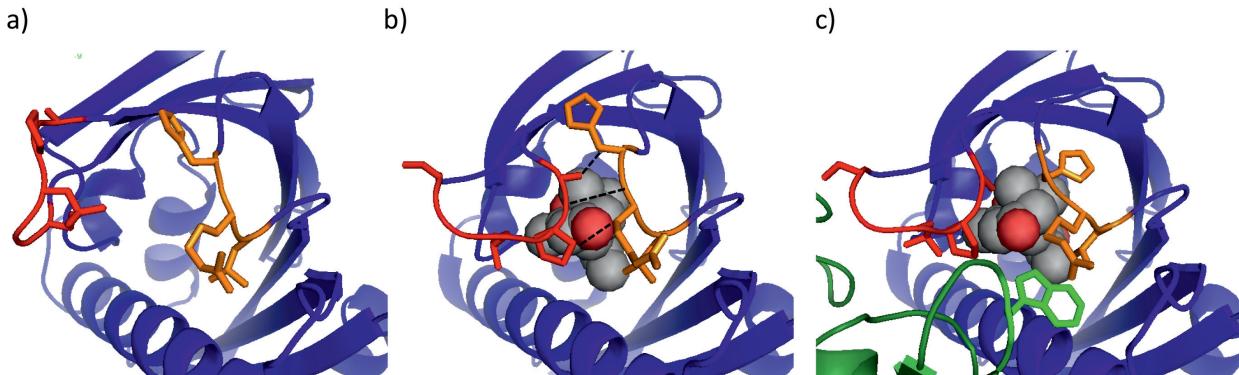


Figure 3. A gate-latch-lock mechanism of ABA sensing and signal relay by the PYL ABA receptor. a) The empty pocket of the ligand-free ABA receptor, represented by apo PYL2 (PDB code: 3KAZ), is guarded by a latch loop (shown in orange) and an open gate loop (shown in red). b) Structure of PYL2–ABA (PDB code: 3KB0) showing that ABA binding induces closure of the gate onto the latch loop. ABA is shown in sphere model. c) PP2C (shown in green) binding inserts a conserved tryptophan to “lock” the closed receptor gate and latch in a stable conformation, shown by the structure of the PYL2–ABA–HAB1 complex (PDB code: 3KB3). In all panels, PYL2 is shown in blue with its gate loop in red and latch loop in orange.

4.3. Signal relay to PP2Cs

The closure of the receptor gate upon ABA binding is functionally important for its signal transmission to the PP2Cs. While PP2Cs do not undergo obvious conformational changes in their interactions with PYLs, the ABA-induced receptor gate closure creates the necessary PYL conformation for PP2C interaction. The ABA-bound PYL protein interacts with PP2C at its closed gate and latch interface (Figure 4). In this interaction, the PP2C inserts a conserved tryptophan indole ring into the receptor pocket, forming a water-mediated network of hydrogen bonds with the receptor gate and latch residues, as well as with the ketone group of ABA (Figure 3b). The PP2C tryptophan thus acts as a molecular lock that further stabilises the receptor gate and latch interactions. This explains the observations of the marked increase in ABA binding affinities of PYLs in the presence of PP2Cs [16, 19, 48, 51]. The formation of the PYL–ABA–PP2C complex inhibits the PP2C activities in two ways. First, the PYL–ABA interacts with the PP2C at the phosphatase catalytic region (Figure 4a), thus competitively blocking substrate (SnRK2) access [56]. Second, a catalytic glutamate residue of the PP2C (E203 in HAB1) is bonded to the serine residue of the PYL gate loop (Figure 4b and 4c), thus impairing the phosphatase catalytic activity [57].

4.4. PP2C inhibition of SnRK2s

In the absence of ABA, PP2Cs bind to and inhibit SnRK2s. The solved structure of the SnRK2.6–HAB1 complex together with biochemical data has provided insights into the mechanisms of how PP2Cs inhibit SnRK2s [56]. In this structure, the phosphatase-kinase interaction occur

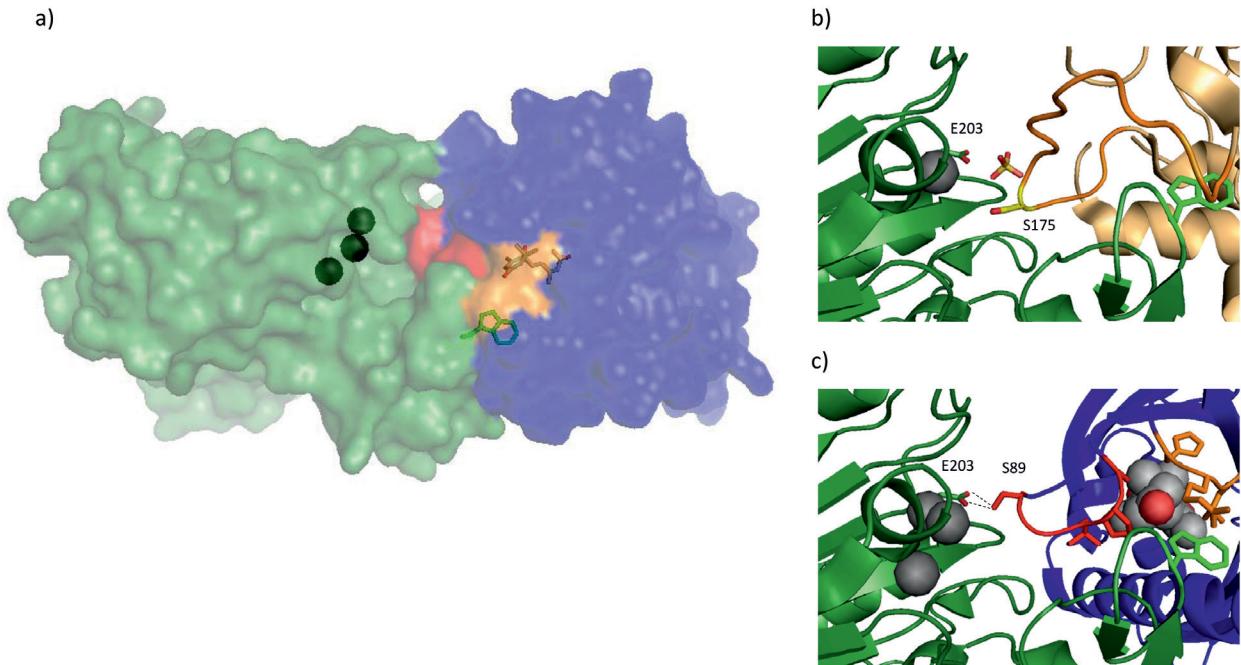


Figure 4. Dual mode of PP2C inhibition by the activated ABA receptor. a) Structure of PYL2–ABA–HAB1 complex (PDB code: 3KB3) in surface presentation showing the steric blocking of PP2C (shown in green, catalytic site marked by Mg²⁺ ions in grey balls) by ABA-bound PYL2 (shown in blue, with its gate and latch in red and orange respectively). ABA is shown in stick model to indicate the ligand pocket. b) Partial structure of the HAB1–SnRK2.6 complex (PDB code: 3UJG), focusing on the catalytic sites of both components. In the phosphatase reaction, a serine residue (S175 of SnRK2.6) in the activation loop of the SnRK2 (SnRK2.6 shown in light orange, with its activation loop in darker orange) is dephosphorylated. A sulphate molecule, mimicking the cleaved phosphate, is shown in stick model. This catalysis requires the PP2C catalytic glutamate (E203 of HAB1) to polarise a water molecule, enabling its nucleophilic attack on the phosphorylated S175 [57]. c) Binding of PYL2–ABA to HAB1 results in the formation of a hydrogen bond between PYL2 S89 and HAB1 E203, thus catalytically inhibiting the PP2C’s phosphatase activity. In all panels, the PP2C tryptophan “lock” is shown as stick model in light green.

through mutual packing of both catalytic sites (Figure 4b and 5a). HAB1 interacts with SnRK2.6 at its PYL-interaction interface and inserts its ABA-sensing tryptophan into the catalytic cleft of SnRK2.6. Unresolved in the crystal structure, extensive evidence indicated the presence of a second interaction interface formed by the highly negatively charged C-terminal ABA box of SnRK2 and a positively charged surface region of PP2C (Figure 5a). Biochemical data has shown that at low PP2C:SnRK2 molar ratio, the kinase activity is reduced but not completely abolished [56]. This partial inhibition occurs by enzymatic dephosphorylation of a critical serine residue in the kinase activation loop. At stoichiometric levels, full inhibition is achieved through the mutual packing of catalytic sites as shown by the crystal structure.

4.5. Autoactivation of SnRK2s

The understanding of how PP2Cs inhibit SnRK2s has provided partial explanations to how SnRK2s gain catalytic activity. PP2C interact with both PYL and SnRK2 at its catalytic region, suggesting that the inhibition of SnRK2 is competed away by active PYL interacting at the same PP2C interface (Figure 5a and 5b). Further biochemical and structural analyses have furnished the complete mechanisms of SnRK2 activation [58-60]. Biochemical data showed

that phosphorylation of a serine residue in the SnRK2 activation loop (S177/176/175 in SnRK2.2/2.3/2.6, respectively) is critical for full kinase activity [61]. Kinases with this serine mutated to alanine are non-phosphorylatable at this position and showed marked decrease, but not complete abolishment of kinase activity, suggesting that unphosphorylated SnRK2s have basal kinase activity [58]. Crystal structures of unphosphorylated SnRK2s have been solved in their active and inactive states, providing structural insights into the mechanisms of the basal activity [58]. The SnRK2 structures revealed a canonical bilobal kinase fold, with a well-ordered SnRK2 box packing closely in parallel to the α C-helix in the N-terminal lobe (schematically illustrated in Figure 5). The structure of the active SnRK2 adopted a closed conformation, with its N-terminal lobe positioned closer to the C-terminal lobe (Figure 5d), whereas that of the inactive SnRK2 exhibited an open conformation (Figure 5c) resembling the structures of active and inactive Snf1 kinases, respectively. The basal kinase activity is attained by the closing of the catalytic cleft, which is regulated by the SnRK2 box interactions with the α C-helix. The SnRK2 box mediates positioning of the α C-helix to form a network of interaction that favours the binding of Mg^{2+} and ATP in the catalytic cleft. Such a positioning enables full kinase activity to be achieved upon autophosphorylation of the activation loop, which can occur intramolecularly or intermolecularly (Figure 5e).

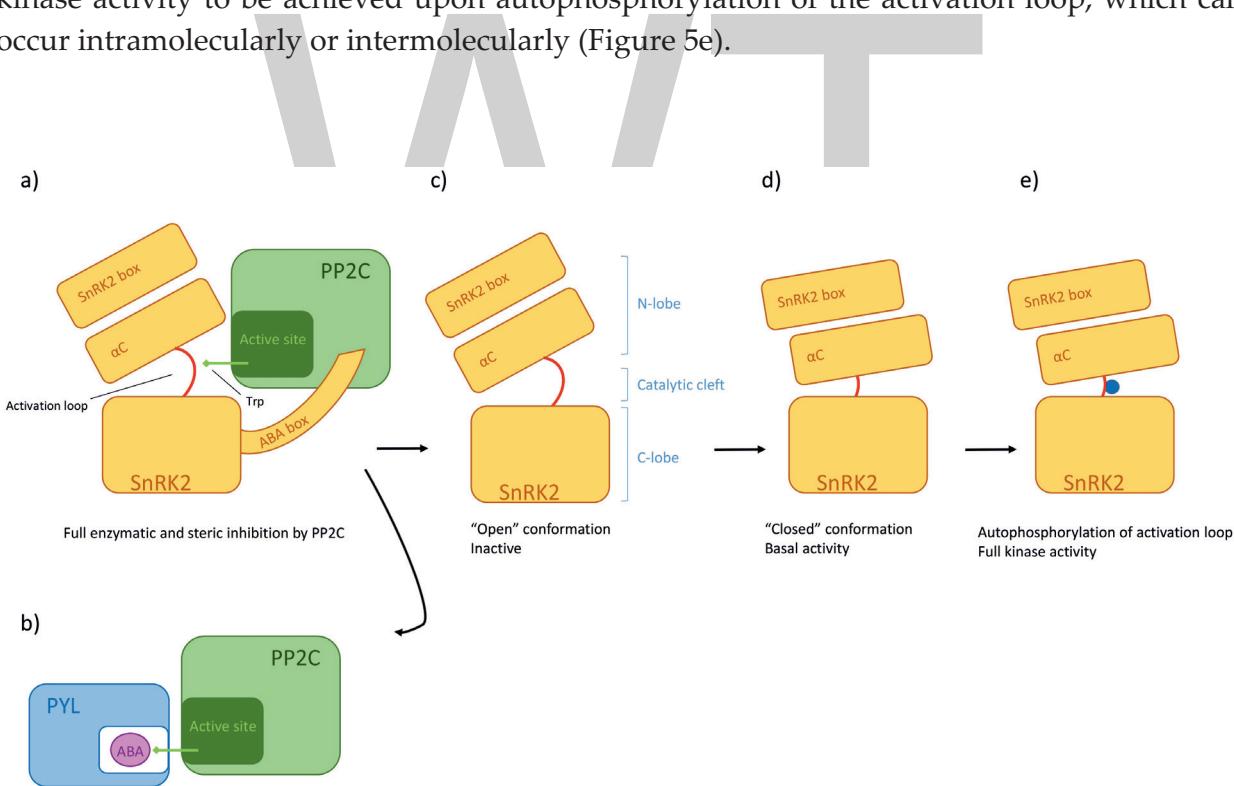


Figure 5. Mechanisms of kinase activation. a) In basal state, PP2C inhibit SnRK2 by enzymatic dephosphorylation of the kinase's activation loop serine, as well as steric inhibition by physically binding to the kinase's catalytic cleft. The two PP2C-SnRK2 interaction interfaces are shown. First is through the mutual packing of active sites as indicated by the kinase's activation loop and the phosphatase's ABA-sensing tryptophan "lock". Second is through the SnRK2's C-terminal ABA box region. b) When activated by ABA, PYL compete with SnRK2 for PP2C interaction at the same PP2C catalytic region. c) and d) When relieved of PP2C inhibition, unphosphorylated SnRK2s spontaneously adopt inactive and partially active states by the opening and closing of the catalytic cleft, mediated by contacts between the SnRK2 box and α C-helix. e) The closing of the catalytic cleft produces interactions that facilitates the autophosphorylation of the activation loop serine residue, which is required to attain full kinase activity.

5. Emerging agricultural biotechnology targeting ABA receptor signalling

The understanding of how plants perceive stress provides the opportunity to develop novel solutions to promote crop survival by boosting stress responses during adverse conditions such as water shortage. As water conservation is one of the physiological effects of ABA, the activation of ABA signalling may also promote water use efficiency in agriculture. The world is facing a crisis of freshwater shortage and the agricultural sector is the largest consumer of the global freshwater resources. Thus, the ability to manipulate ABA responses has immense value, promoting both water conservation and food productivity. ABA responses may be manipulated by transgenic approaches involving the overexpression of the effectors of ABA signalling, the structure-guided development of high efficacy agonists, or a combination of these methods.

5.1. Transgenic approach

Having understood the roles and mechanisms of the key players in the core ABA signalling, one approach to enhance ABA sensitivity is to generate transgenic plants overexpressing the effectors of ABA signalling. While the dimeric PYL members, PYR1, PYL1, and PYL2 require ABA for their activation, the monomeric receptors, PYL5-10, show ABA-independent constitutive activity which is greatly enhanced in the presence of ABA [46]. Thus, the constitutively active monomeric receptors are thought to be suitable candidates for transgenic overexpression to enhance ABA sensitivity. This idea is supported by the observation that transgenic Arabidopsis overexpressing PYL5 showed enhanced ABA sensitivity and increased drought stress tolerance [19]. Similar results have been shown in transgenic Arabidopsis overexpressing PYL13 [53], which has shown ABA independent inhibition of PP2CA [54]. In rice (*Oryza sativa*), the constitutive expression of OsPYL, the rice orthologue of Arabidopsis PYL, has also shown to improve drought and salt stress tolerance [62].

With the known structural mechanisms, genetic modifications of PYL may be incorporated to further enhance their activity. The constitutive expression of a PYL2 mutant designed to stabilise PYL-PP2C interactions has shown increased ABA signalling in transgenic Arabidopsis seeds [63]. However, the expression of the mutant receptor has not been detected in vegetative tissues, thus precluding further analyses in this study. In another study, overexpression of a mutant PYL4 receptor (PYL4^{A194T}), which showed ABA-independent inhibition of PP2CA in vitro, resulted in enhanced drought tolerance in the transgenic plants [64]. However, as ABA is a negative growth regulator, a drawback of constitutively activating ABA responses is the impairment of growth under normal conditions, thus affecting overall yield. Approaches that allow inducible activation of ABA responses only under stressful conditions may be useful to overcome this problem.

Alternatively, the heterologous expression of SnRK2s of wheat (*Triticum aestivum* L.), TaSnRK2.4, and TaSnRK2.8 in Arabidopsis has been tested and the transgenic plants showed enhanced tolerance to drought, salt, and cold stresses [65, 66]. Similarly, transgenic Arabidopsis overexpressing the maize SnRK2 orthologue, ZmSAPK8, exhibited increased tolerance to salt stress [67]. In these studies, the heterologous expression of members of the SnRK2s did

not seem to retard the normal plant growth under unstressed conditions, thus appearing to be a potential approach for the development of stress-tolerant transgenic crops.

5.2. Agonist design

The direct application of ABA in the fields has been shown to promote drought stress tolerance. However, the widespread use of ABA in agriculture is limited due to its chemical instability and difficulty to synthesise. Therefore, there has been much research focus on the development of ABA analogues that are able to elicit the effects of ABA and exhibit better chemical stability.

Pyrabactin is a synthetic seed germination inhibitor that mediates a selective part of ABA's activities. Being a selective ABA agonist, pyrabactin has been used to overcome the problems of genetic redundancy in the identification of ABA receptors [17]. Although the direct application of pyrabactin is not practical for agriculture use, as its effects are most strongly in seeds rather than in vegetative tissues, the study of ABA receptors bound to pyrabactin has provided insights into the design of ABA receptor agonists. While ABA is a pan-agonist of all PYL members, pyrabactin selectively activates few members, including PYR1 and PYL1. Conversely, pyrabactin acts as an antagonist in PYL2. Comparisons between the structures of ABA-bound PYL, pyrabactin-activated PYL and pyrabactin-antagonised PYL complexes have revealed the ligand-receptor interactions that are important to induce the closed gate conformation necessary for receptor activation [68-71]. In the pyrabactin-activated receptor (PYR1 or PYL1) structures, pyrabactin interacts with receptor pocket residues in a similar manner as ABA, with its sulphonamide group forming a water-mediated hydrogen bond with the conserved lysine residue and its naphthalene double ring interacting with the receptor gate residues, producing the close-gate conformation (Figure 6b). These features provided a framework for the development of novel ABA agonists. On this basis, Melcher et al. screened virtual chemical libraries for compounds containing the naphthalene-1-sulphonamide group and computationally docked them into the known PYL structures [69]. In vitro screening of top candidates identified four compounds with efficacies comparable to that of pyrabactin.

Examination of the structure of the pyrabactin-antagonised PYL2 receptor revealed a flip in the orientation of pyrabactin relative to that in the pyrabactin-activated receptor structures (Figure 6c). In the antagonist position, the naphthalene double ring and pyridine ring are in reversed order, placing the smaller pyridine ring towards the pocket entrance in a distance too far to interact with the receptor gate. Therefore, pyrabactin antagonises PYL2 by occupying the receptor pocket while being unable to produce the closed gate conformation. With this knowledge, an agonist termed AM1/quinabactin was designed with a similar scaffold as pyrabactin but with the sulphonamide group in reversed orientation [72, 73]. The structures of PYL2 bound to AM1/quinabactin showed that the AM1/quinabactin is oriented with its double ring facing the receptor gate, producing a closed gate conformation (Figure 6d). Furthermore, AM1/quinabactin showed potent in vitro efficacies in PP2C interaction, ability to promote drought tolerance when exogenously applied to plants and higher stability than ABA when exposed to mild UV [72, 73]. Therefore, AM1/quinabactin emerged as a highly promising candidate to be further evaluated for agricultural use.

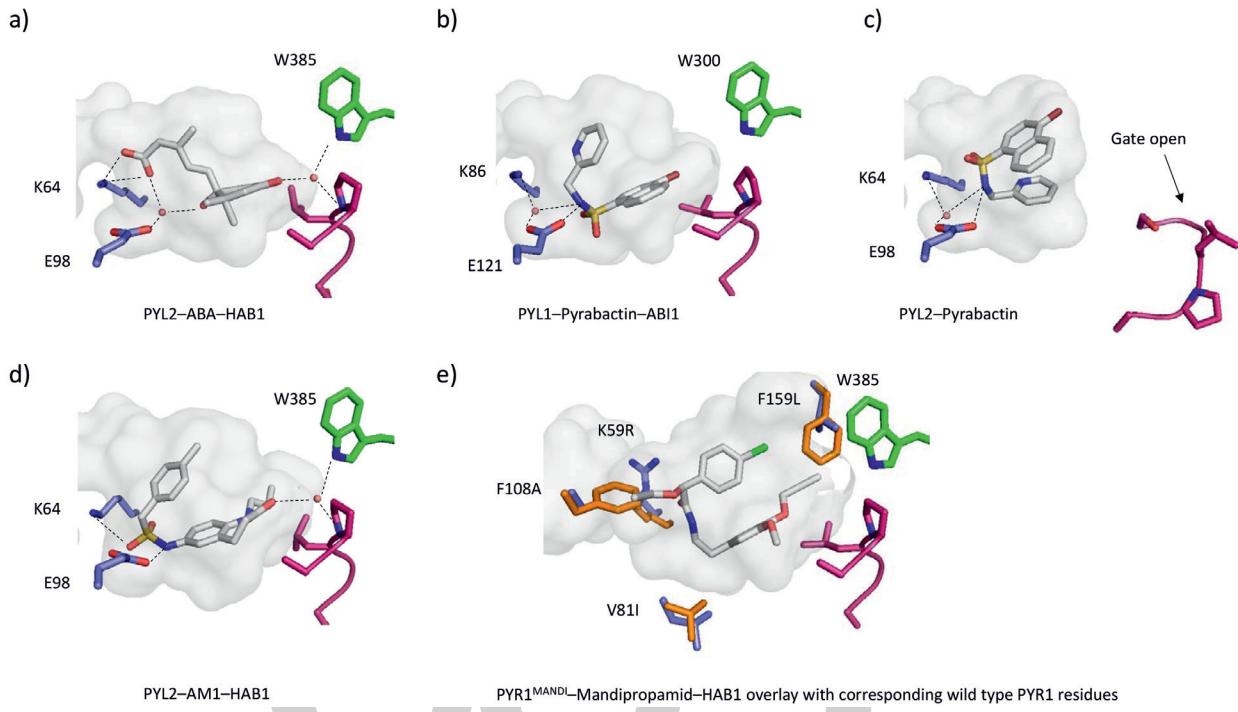


Figure 6. Mode of receptor binding by various ligands. Ligand binding pockets of a) PYL2–ABA–HAB1 structure (PDB code: 3KB3), b) PYL1–Pyrabactin–ABI1 (PDB code: 3NMN), c) PYL2–Pyrabactin (PDB code: 3NMH), d) PYL2–AM1–HAB1 (PDB code: 4LG5), and e) PYR1^{MANDI}–Mandipropamid–HAB1 (PDB code: 4WVO). PYL ligand pockets are shown as surface presentation in light grey. In (a) to (d), the conserved lysine and glutamate residues that anchor the ligands to the inner end of the receptor pocket are shown as stick models in blue. In all panels, receptor gate loop residues are shown in red, while the conserved PP2C tryptophan “lock” is shown as a stick model in green. In (c), pyrabactin antagonises the PYL2 receptor, leaving the receptor gate in an open conformation. In (d), the four mutant PYR1^{MANDI} residues are shown in blue, while the corresponding wild type residues from the PYR1–ABA–HAB1 (PDB code: 3QN1) structure are shown in orange.

5.3. Orthogonal receptor-ligand approach

Although the discovery of AM1/quinabactin has shown promising results, pushing a new chemical into the market can be a long and costly process as the potential effects on human health and the environment need to be thoroughly assessed. To bypass such a process, an alternative solution is to make use of currently approved agrochemicals. Park et al. has demonstrated that the ABA receptor can be engineered to be activated by existing agrochemicals [74]. This effort has identified a hexuple mutant PYR1^{MANDI} (PYR1(Y58H/K59R/V81I/F108A/S122G/F159L)) that showed strong PP2C inhibition with the agrochemical mandipropamid at nanomolar sensitivity. To understand the mode of the orthogonal receptor-ligand interactions, the crystal structure of a quadruple mutant PYR1(K59R/V81I/F108A/F159L), which contains 4 of the 6 mutations of PYR1^{MANDI} and yielded higher quality crystals than PYR1^{MANDI}, has been solved in complex with mandipropamid and HAB1 (Figure 6e). The crystal structure revealed that the F108A/F159L mutations created more space in the receptor pocket to fit the larger ligand. The arginine of R59 forms a hydrogen bond with the amide carbonyl of mandipropamid, mimicking the interaction between the carboxylate group of ABA with K59 of wild type PYR1. In vivo studies of transgenic *Arabidopsis* constitutively express-

ing PYR1^{MANDI} showed enhanced drought survival with mandipropamid treatment, thus demonstrating the feasibility of such an approach.

6. Conclusions

The identification of PYL proteins as ABA receptors has placed a crucial piece of the puzzle into the previous knowledge of the core ABA signalling pathway. As such, the early efforts in the discovery of PYL proteins and the elucidation of their structural mechanisms have been recognised as one of the breakthroughs of the year 2009 by *Science* and *Science Signaling* journals [75, 76]. Subsequently, further structural studies have rapidly emerged to provide a comprehensive understanding of the molecular mechanisms of the entire core ABA signalling pathway, which has led to preliminary developments of novel approaches targeting at ABA signalling for the agricultural enhancement of abiotic stress tolerance. Using several lines of approach, these studies have demonstrated the ability to target ABA signalling for improving stress tolerance. While several studies have shown promising data at the preliminary stage, further testing in crop plants will be necessary to evaluate their agricultural feasibility. Having progressed rapidly since the initial discovery of the PYL proteins, further breakthrough advances in the agricultural improvement of abiotic stress tolerance seems highly conceivable and is much awaited for.

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Rootstock Breeding for Abiotic Stress Tolerance in Citrus

Berken Cimen and Turgut Yesiloglu

Abstract

Citrus species are the most widely produced fruit crops in the world. Citrus fruits are mainly produced in coastal areas in several countries as well as Mediterranean region, and production in these regions is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity. There is extensive genetic diversity in citrus which provides several materials to be used as rootstocks against abiotic stress. In this work, we tried to provide an overview of the abiotic stresses in citrus by combining literature with our studies, role of citrus rootstocks commercially used against abiotic stresses and rootstock breeding in citrus.

Keywords: Citrus, rootstock, salinity, alkalinity, drought, breeding

1. Introduction

Citrus is the most important tree fruit crop in the world, and citrus fruits are regarded as major household items in more than 100 countries around the world as well as the world juice industry which is also led by citrus juices. Citrus industry is regarded as a leading industry in some regions, such as the mountainous regions of China and coastal plains in several countries, such as California and Florida in USA, Valencia in Spain, and Adana in Turkey. According to the data published in 2013, the world's total citrus fruit production is 135.761.181 tons [1], which consists of 71.445.352 tons of oranges (*Citrus sinensis* (L.) Osb.), 28.678.213 tons of mandarins (*Citrus reticulata* Blanco), 15.191.482 tons of lemons (*Citrus limon* Burm. F.) and limes (*Citrus latifolia* Tan. and *Citrus aurantifolia* Swingle), 8.453.446 tons of grapefruits (*Citrus paradisi* Macf.) and pummelos [*Citrus maxima* (Burm.) Merr.], and 11.992.686 tons of other citrus fruits (Figure 1).

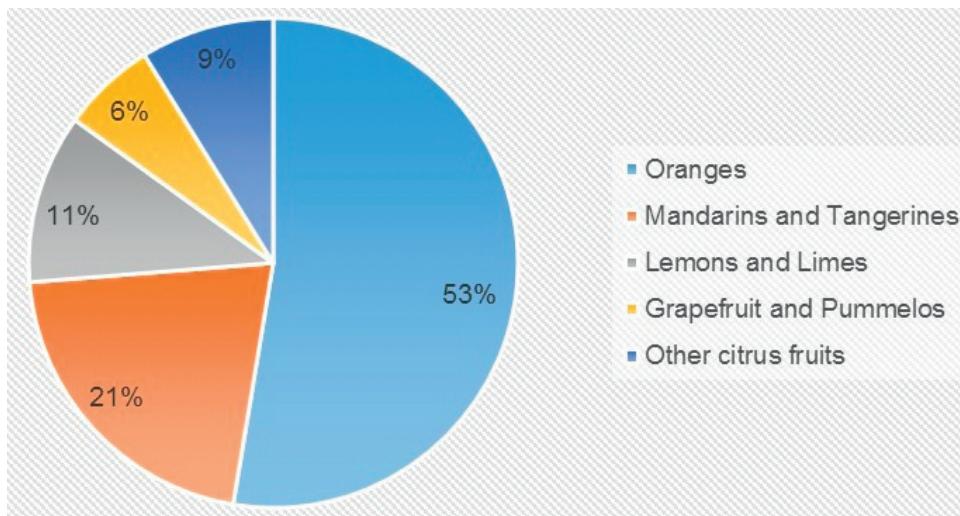


Figure 1. World's citrus production quantity (tons)

The role of citrus fruits in providing nutrients and medicinal value has been recognized since ancient times. Citrus fruits, belonging to the genus *Citrus* of the family Rutaceae, are well known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C as per recommended dietary allowance. In addition to ascorbic acid, these fruits contain several phytochemicals, which play the role of nutraceuticals, such as carotenoids (lycopene and β -carotene), limonoids, flavanones (naringins and rutinoside), and vitamin B complex and related nutrients (thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, biotin, choline, and inositol). These substances greatly contribute to the supply of anticancer agents and other nutraceutical compounds with antioxidant, inflammatory, cholesterol, and allergic activities, all of them essential to prevent cardiovascular and degenerative diseases, thrombosis, cancer, atherosclerosis, and obesity. In spite of these beneficial traits, there is still a major need to improve fruit quality to meet current consumers' demands [2, 3].

Being vegetatively propagated, a citrus tree is normally composed of the rootstock and scion. Rootstocks play an important role in the rapid development of citrus in the world as well as breeding new cultivars. The necessity of using rootstocks for citrus fruits is to have a profitable production against some limiting factors such as climate, bad soil conditions, and diseases. Besides these factors, the use of citrus rootstocks provides a large number of choice to the growers to increase fruit quality and yield, obtain early fruiting and uniform cropping, avoid juvenility, controlling the tree size, have the opportunity for high-density planting, etc. These factors give many economic important advantages to the growers and, as a result, the citrus fruits are the most produced fresh fruits in the world for several decades [4]. Choosing a rootstock is an important decision, and local climatic and soil conditions are important factors in rootstock selection. Although any citrus variety can be used as a rootstock, some of them are better suited to specific conditions than the others [5, 6]. Some characteristics in a desirable citrus rootstock should be listed such as a good adaptation to all kinds of soils, tolerance to salinity, iron chlorosis, flooding, drought, high affinity with commercial species/cultivars, high yields of good fruit quality, reduced tree size, resistance to citrus tristeza virus (CTV), resist-

ance to citrus blight, resistance to fungal diseases affecting citrus (*Phytophthora* spp., *Armillaria mellea*, etc.), and resistance to nematodes.

Citrus production is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. These stresses can severely influence growth and development of both rootstocks and/or scions of citrus trees, thus reducing both fruit production and fruit quality. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity [7]. Soil salinity is a major factor reducing crop production among the world as well as citrus production. Responses to salinity in terms of citrus production are affected by the amount of irrigation, climate, soil type, and fertilization [8]. Citrus rootstocks differ in terms of their tolerance to salinity conditions; many soils and sources of water contain high amounts of salts that can inhibit the growth and yield salt-sensitive citrus [9]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls, and production of yellow, chlorotic leaves resulting as a decrease in fruit yield and quality [10, 11, 12]. Drought is considered as the principal factor that limits global agricultural production, among environmental constraints. Species greatly differ in the ability to overcome water deficiency. Drought in citrus trees causes reductions in stomatal conductance (g_s), leaf transpiration rate (E), and net CO_2 assimilation; decreases fruit quality and yield in long-term periods of stress; and increases fruit abscission [13].

Although the sour orange has many excellent horticultural advantages in terms of abiotic stress, it has a very important disadvantage for its susceptibility to citrus tristeza virus. This problem has severely reduced the use of this rootstock in many places especially the Western Mediterranean. Castle and Gmitter [14] reported that sour orange no longer has a secure place in today's rootstock portfolios because of its susceptibility to CTV. Castle [15] indicated that sour orange is an excellent rootstock for areas free of CTV. Ollitrault et al. [16] reported that the arrival of tristeza radically called into question of using sour orange in the Mediterranean area, whereas it had been almost the only rootstock in the region.

The need to produce stress-tolerant crops was evident even in ancient times [17]. Searching rootstock alternative to sour orange keeps on all over the world. In addition, it is a known fact that every rootstock cannot be used in every ecology. Due to the limiting effects of different ecological factors and diseases, a rootstock which is suitable for a country could not succeed in another one. Accordingly, the rootstocks that will be offered as alternative to sour orange is supposed to show some characteristics such as tolerance to lime-induced iron chlorosis in calcareous soils, tolerance or resistance to *Phytophthora citrophthora*. In addition to these, the alternative rootstocks should have CTV tolerance which does not exist in sour orange. On the contrary, all the required traits are present in the citrus germplasm. For instance, *Poncirus trifoliata* has tolerance to mainly biotic stress and cold hardness and some *Citrus* species have

adaptation to both abiotic and biotic stresses. However, the complexity of citrus biology and genetics makes it difficult to combine them through traditional breeding.

Consequently, in this chapter, we try to provide an overview of the abiotic stresses in citrus, role of citrus rootstocks commercially used against abiotic stresses, and rootstock breeding for tolerance to abiotic stress.

2. Types of abiotic stresses in citriculture

Many different citrus genotypes are commercially grown in a wide diversity of soil and climatic conditions; therefore, trees are subjected to important abiotic and biotic stresses that limit the production and, in some instances, the use of certain rootstocks and varieties [18]. Citrus trees are subjected to several abiotic constraints such as acid, alkaline, and salty soils; flooding and drought; and freezing and high temperatures.

Related to the global warming, drought problems have occurred in many countries. In addition, salinity became a major cause for citrus production in the coastal regions of Mediterranean by increasing the use of fertilizers and decreasing precipitations. Salinity and drought in the calcareous soil of Mediterranean region can lead to major problems in citriculture in terms of fruit yield and quality. Thus it is necessary to breed new rootstocks that are genetically tolerant to abiotic stress conditions and alternatives to existing rootstocks.

In a recent work, we have tried to handle three common abiotic stresses (salinity, drought, and alkalinity) occurring in citriculture especially in the coastal Mediterranean region by combining the literature with the studies conducted at Çukurova University.

2.1. Salinity

Salinity is a major environmental factor affecting the performance of many crop plants and reducing agricultural productivity [19, 20]. It is estimated that more than a third of all of the irrigated soils in the world is affected by salinity. The loss of farmable soils due to salinization is directly in conflict with the needs of the world population which is increasing continuously. Salt stress is a major stress problem in arid and semiarid regions and irrigated areas. Almost 7% of the world land area, 20% of the cultivated land, and nearly half of the irrigated land are affected by high salt concentrations [21].

Salinity affects the crop during both the vegetative and the reproductive stage and therefore causes reduction in plant growth and development with low water potential in the root medium (osmotic effect), too high internal ion concentration (ion excess/toxicity), and nutritional imbalance by depression in uptake and/or shoot transport (ion deficiency). Most of the salt stress in nature is due to sodium salts, particularly NaCl [22, 23]. High concentrations of Na^+ and Cl^- in the root medium saturation depress nutrient-ion activities and produce extreme ratios of $\text{Na}^+/\text{Ca}^{2+}$, Na^+/K^+ , $\text{Ca}^{2+}/\text{Mg}^{2+}$, and $\text{Cl}^-/\text{NO}_3^-$ [24]. As a result, plants become susceptible to osmotic and specific ion injury as well as nutritional disorders that may result in reduced yield and quality. These processes may be occurring at the same time, but whether they

ultimately affect crop yield and quality depends on the salinity level, composition of salt, exposed period to salinity, the crop species and cultivars, the growth stage of plants, and a number of environmental factors [25, 26, 27, 28]. When the salt concentration reaches a harmful level for plant growth, a salinity condition is said to have developed. The degree to which growth and normal metabolism can be maintained is described as salt tolerance. Salt tolerance of vegetable crops varies considerably among species and depends upon the cultural conditions under which the crops are grown. Soil, water, plant, and environment can affect the salt tolerance of a plant. Therefore, plant response to a given salt concentration cannot be predicted on an absolute basis but on relative performance [29].

The effects of salinity on plants are evidenced by a severe reduction in plant growth and yield and, if the saline conditions persist, plant death can occur [30]. Salinity causes a deficiency of water in plant tissue, and low water potential reduces growth by inhibiting cell division and cell expansion [31]. The reduction in growth is mainly due to an osmotic effect of the accumulation of salts near the root zone, whereas the buildup of toxic saline ions in plant tissues is responsible for the progressive impairment of several physiological processes [20].

Osmotic effects resulting from salinity may cause disturbances in the water balance of the plant, including a reduction of turgor and an inhibition of growth, as well as stomatal closure and reduction of photosynthesis [32, 33]. The primary effect of high salt concentration in plants is stomatal closure. This causes a low transpiration rate and reduces the CO₂ availability for photosynthesis [34]. Hussain et al. [35] indicated that salinity reduced the photosynthetic availability of some citrus species and genera. As a result, plants become susceptible to osmotic and specific ion injury as well as to nutritional disorders that may result in reduced yield and quality.

Many researchers so far have reported citrus trees as salt-sensitive plants [30, 36, 37]. Salinity reduces citrus tree growth and fruit yield [9]. Growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl⁻ and Na⁺ in leaves are the main problems that are caused by salinity stress [37]. Also, salt stress has a dramatic impact on the citrus industry by decreasing the growth of trees and fruit yield and quality. Salinity may also cause nutrient deficiencies or imbalances, due to the competition of Na⁺ and Cl⁻ with nutrients such as K⁺, Ca²⁺, Mg²⁺ and NO₃⁻. In addition to osmotic effect, high K⁺ concentration in a salinized nutrient solution increased the absorption of Cl⁻ citrus roots. Salt tolerance in citrus has been linked to the exclusion of toxic ions from the shoot [38]. Thus, citrus rootstocks have a great influence on the amount of Cl⁻ and/or Na⁺ accumulated in the foliage of grafted trees [30]. For instance, in Fino lemon trees, sour orange rootstock is considered a good Cl⁻ and Na⁺ excluder, whereas the *Citrus macrophylla* rootstock is a Cl⁻ and Na⁺ accumulator [39].

Citrus trees, under salinity stress, suffer growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl⁻ and Na⁺ in leaves [37]. Depending on the soil type, irrigation method, and frequency, the soil solution salinity might also rise several fold between irrigations. Continual improvement of rootstocks and/or scions will be necessary to sustain irrigated citrus in increasingly salinized environments [30].

Sour orange is one of the most frequently used rootstocks in Mediterranean countries. It is known to be tolerant to salinity and calcareous soil among citrus rootstocks. However, it is highly susceptible to tristeza disease [37], that the disease should take into consideration the citriculture in Turkey and other countries which use sour orange as rootstock owing to be threatened by it. Genetically improved with favorable agronomical characteristics, such as resistance to pests and diseases such as the citrus tristeza virus, and salinity tolerance, rootstocks may be a long-term approach. Hence, screening studies based on physiological responses of genotypes to salinity stress should be used. In addition, continuous improvement of rootstocks is necessary to sustain cultivating citrus trees under salinized environments.

Yesiloglu et al. [40] established a screening study for the physiological evaluation of global tolerance to salinity rootstock collection of Çukurova University in the frame of the CIBEWU project, No: 015453. They screened 29 different genotypes that can be used as citrus rootstock under salinity stress assessed by several growth and physiological parameters such as fresh and dry weights of shoot and root; leaf chlorophyll concentration; and fluorescence, chloride, and Na content. High concentration of Cl and/or Na in the leaves of Citrus has been frequently related to disturbances in nutrition, gas exchange, and water relations. Unpublished data of the project regarding the genotypes used in the screening study and Cl⁻ and Na⁺ concentrations in root and leaves of the genotypes are presented below. 34-12 N citremon, 4475 SRA citrumelo, CRC 4475 Swingle citrumelo, *Severinia buxifolia* have accumulated higher chloride in leaves, while Tuzcu Cleopatra mandarin, Gou Tou, Rangpur lime, CRC 02 Volkameriana, Tuzcu 31-31 sour orange have lower chloride in leaves. Rubidoux trifoliolate with trifoliolate hybrids and *Severinia buxifolia* were poor Cl⁻ excluder. It was found that Tuzcu Cleopatra mandarin and Rangpur lime are the best Cl⁻ excluders. Gou Tou, Antalya Cleopatra mandarin have the lowest amount chloride in the roots, whereas *Citrus ichangensis*, Benecke trifoliata, Volkameriana, 08 A 3015 Rubidoux, and SRA Pomeroy have the highest values. According to the results, Tuzcu Cleopatra mandarin, Rangpur lime, Gou Tou, and Antalya Cleopatra mandarin were found to be the most tolerant to salt stress. *Severinia buxifolia*, CRC-4475 Swingle citrumelo, Local trifoliata, and Benecke trifoliata were the most sensitive group to salt stress. Data were evaluated by using modified "weighted-ranking" method based on the parameters of chlorophyll fluorescence (F_v'/F_m'), leaf chlorophyll concentration by SPAD readings, leaf Cl and Na concentrations, leaf K and Ca concentrations, root K and Ca concentrations, growth parameters (shoot and root fresh and dry weights, shoot length, and leaf number), and visual ratings of leaf chlorosis. A classification which belongs to the screening study for salinity stress was performed as a result of the work and reported as follows in Table 1 (unpublished data). Genotypes were classified as very sensitive, sensitive, acceptable, tolerant, and very tolerant to salinity.

Khoshbakht et al. [41] reported that the effects of salinity on photosynthesis range from the restriction of CO₂ diffusion into the chloroplast, via limitations on stomatal opening mediated by shoot- and root-generated hormones, and on the mesophyll transport of CO₂, to alterations in leaf photochemistry and carbon metabolism. The authors conducted a study and investigated the NaCl effects on gas exchange parameters of nine citrus rootstocks and reported that sour orange and Cleopatra mandarin were the rootstocks most tolerant to salinity of all the

Genotypes	Tolerance to salt stress
Rubidoux trifoliate 08A 30.15	2
Citremon 34 12 N	1
Citrumelo 4475 SRA	1
AREC Swingle citrumelo	3
Antalya Cleopatra mandarin	3
Benecke trifoliate	2
C-35 Citrange	4
Swingle citrumelo 4475 CRC	1
Volkameriana CRC 01	4
Volkameriana CRC 02	4
Citrumelo CRC 1452	3
Carrizo citrange	3
<i>Citrus ichangensis</i> CRC	3
<i>Citrus sulcata</i>	3
<i>Citrus sunki</i>	3
Macrophylla	2
Rangpur lime	5
Gou Tou sour orange SRA 506	4
Pomeroy trifoliate SRA	1
Sacaton citrumelo	4
<i>Severinia buxifolia</i> SRA	1
Smooth Seville sour orange	4
Taiwanica	4
Troyer citrange	3
Tuzcu 31-31 sour orange	4
Tuzcu 891 sour orange	4
Tuzcu Cleopatra mandarin	5
Volkameriana	4
Local trifoliate	2

*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

Table 1. Classification of rootstocks in collection of Çukurova University in respect of salinity tolerance

nine citrus rootstocks studied. Also, Cimen et al. [42] determined that the tolerances of Sarawak bintangor, Shekwasha, Fuzhu and Cleopatra mandarin to salt stress were determined by investigating the photosynthetic parameters and significant salinity effects on the photosynthetic performances of these rootstocks were reported (Figure 2).

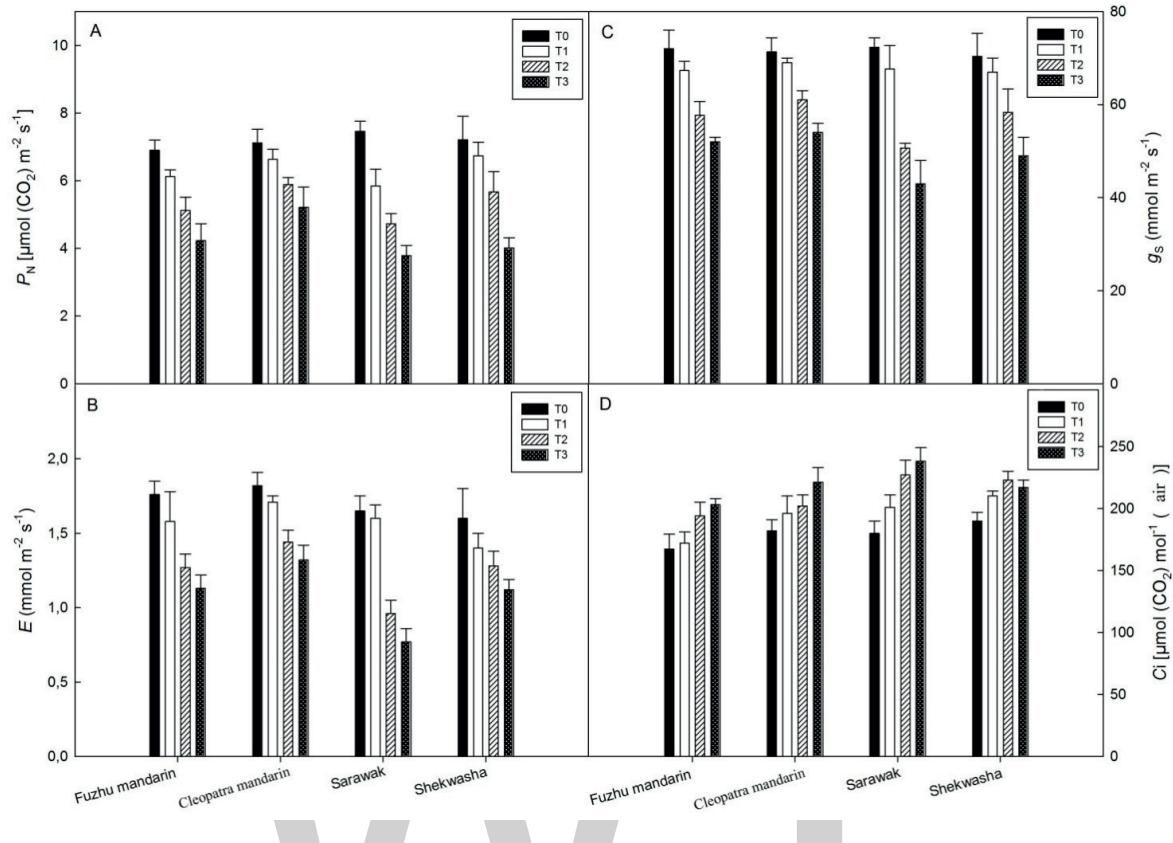


Figure 2. Effects of different salinity levels on photosynthetic rate (A), transpiration rate (B), stomatal conductance (C), and initial CO_2 concentrations (D) of four genotypes. The bars indicate the standard deviation. (T0 = 0 mM NaCl, T1 = 50 mM NaCl, T2 = 75 mM NaCl, T4 = 100 mM NaCl). Data presented from Cimen et al. [42].

2.2. Alkalinity

It is well known that iron is an essential micronutrient for all higher plants including citrus. Most of the iron existing in rhizosphere cannot be taken up by plants because iron is highly insoluble. Two main working hypotheses have been put forward for this chlorosis. In the first working hypothesis, the main cause of Fe deficiency chlorosis is thought to be the inhibition of Fe acquisition by HCO_3^- in the rhizosphere. For the second hypothesis, Fe inactivation in the leaf apoplast by an alkalization process properly noted by the “distant effect” of HCO_3^- is thought to be the main trigger for Fe deficiency chlorosis in leaves [43]. The Mediterranean Basin is characterized by the prevalence of calcareous surface horizon. In these soils, iron (Fe) chlorosis can lead to diminished yields and even plant death, particularly in semiarid areas where irrigation water has high bicarbonate contents, soil pH is high (7.0–9.0), and organic matter content is low [44]. Citrus production is increasing throughout the Mediterranean countries and more and more citrus orchards are being planted on marginal soils. Mediterranean countries have a suitable climate for citrus production, but it is estimated that 20–50% of fruit trees grown in the Mediterranean basin suffer from iron (Fe) deficiency. The most prevalent cause of Fe deficiency in this region is the presence of high levels of carbonate ions in calcareous soils, characterized by a high pH [45]. These soils often have more than 20% of calcium and magnesium carbonates and are strongly buffered, with a pH between 7.5 and 8.5

[11]. Fe uptake is highly dependent on soil pH, and Fe activity in solution decreases 1000-fold for each pH unit rise to reach a minimum within the range from 7.4 to 8.5 [46]. Leaf Fe chlorosis in plants is an old problem occurring in areas of calcareous and/or alkaline soils. Yield reductions from Fe-induced leaf chlorosis have been found in tomato, raspberry, kiwifruit, pineapple, vines, and citrus [47]. Moreover, the severity of leaf chlorosis and the differential behavior of genotypes can be determined by the chlorophyll concentration in leaves [11, 46, 48]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls and producing yellow, chlorotic leaves. The loss of pigmentation is caused by decreased chlorophyll content in chloroplasts. This negatively affects the rate of photosynthesis and, therefore, the development of biomass. Fe deficiency affects the physiology and biochemistry of the whole plant, as Fe is an important cofactor of many enzymes, including those involved in the biosynthetic pathway of chlorophylls [10, 11].

The use of rootstocks in fruit production includes not only a stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metal, nutrient stress, water stress, and alkalinity [7]. Recent studies showed that citrus rootstocks had different tolerance levels to iron deficiency [48, 49]. Studies emphasized that high pH conditions reduced iron uptake in citrus rootstocks [48, 49, 50, 51, 49]. Also, rootstocks affect tree growth, fruit quality, and yield [15, 48, 52]. Moreover, scion behavior depends in part on the rootstock-induced effects on leaf gas exchange [53]. González-Mas et al. [53] indicated that in calcareous soils, citrus production depends on the availability of suitable rootstocks that are tolerant to low Fe soil conditions. Studies have found that "Volkameriana," and "sour orange" plants were tolerant; "Carrizo and Troyer" citranges were intermediate, whereas the "*Poncirus trifoliata*" rootstock was more sensitive to iron chlorosis [46, 48, 49, 51, 54]. In addition, Cimen et al. [55] indicated that Young "Navelina" orange trees budded on Tuzcu 31-31 sour orange, and Gou Tou sour oranges performed best under Fe deprived conditions in plant growth chamber. Navelina on Volkameriana and Cleopatra mandarin was moderate; C-35 citrange and local trifoliate were poorly adapted to lime-induced Fe deficiency (Figure 3).

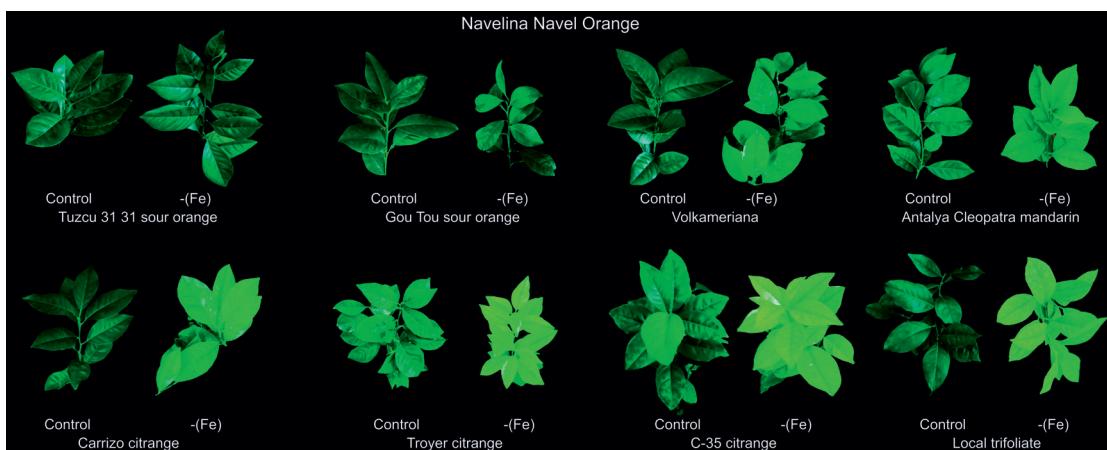


Figure 3. Response of Navelina Navel orange budded on eight different rootstocks in response to iron deficiency. Data presented from Cimen et al. [55].

Among physiological processes, photosynthesis is the basic determinant of plant growth and productivity, and the ability to maintain the rate of carbon assimilation under environmental stress is of fundamental importance to plant production [56]. Since Fe catalyzes chlorophyll biosynthesis [57], it would be expected to promote the photosynthetic rate (*Pn*) while Fe deficiency to reduce it [58, 59]. Most of the knowledge concerning the effect of Fe deficiency on the photosynthetic parameters has been obtained with annual plants. However, relatively few studies have focused on the consequences of induced Fe deficiency on photosynthesis in evergreen fruit trees and especially in citrus. On the contrary, Fe is a component of several metalloenzymes, including peroxidase and catalase. Although both enzymes could be used as biochemical indicators of Fe availability in citrus [36], there are contradictory reports concerning the effect of Fe deficiency on catalase and peroxidase activity [60]. Cimen et al. [55] reported that at sufficient Fe supply, plants had higher activity of catalase (CAT) than the plants with Fe deprived conditions. Slight decreases were recorded on the Navelina orange leaves of Tuzcu 31-31 and Gou Tou sour oranges, while decreases were remarkable in the leaves of C-35 citrange and TGK0633 (obtained by a selection of trifoliate orange in Turkey) under short supply of Fe. In addition, Navelina leaves of C-35 citrange and TGK0633 displayed maximum decreases in APX (ascorbate peroxidase) activity, similarly CAT activity. There were no significant APX activity decreases in the leaves of Tuzcu 31-31, Gou Tou sour oranges, Volkameriana, and Antalya Cleopatra mandarin (Figure 4).

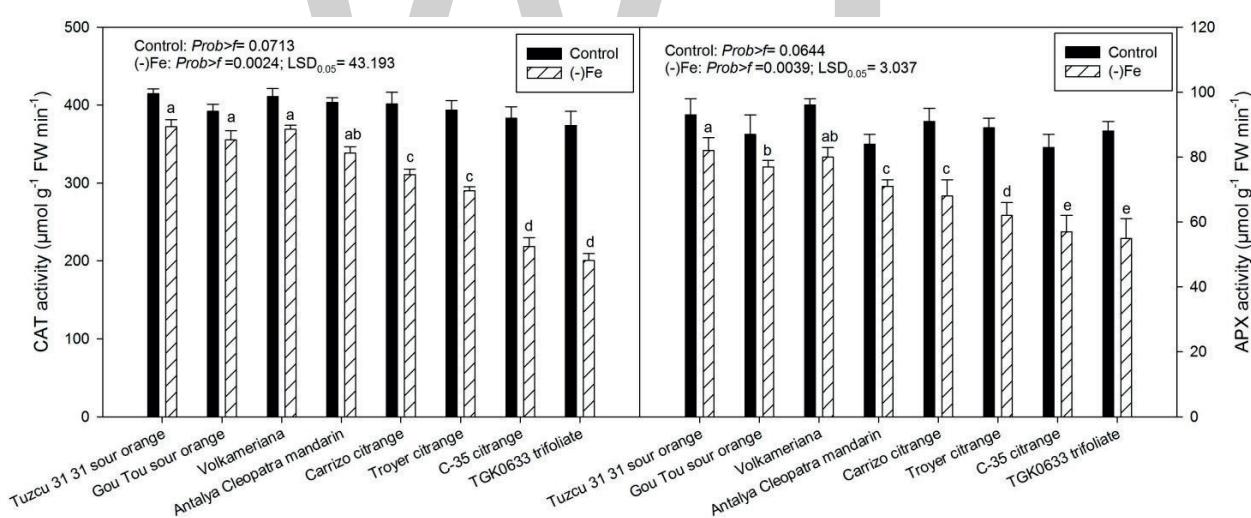


Figure 4. Catalase and ascorbate peroxidase activities of Navelina leaves of different rootstocks under Fe sufficient and deprived conditions. The bars show the standard deviation. Data presented from Cimen et al. [55].

The intensity of iron chlorosis can be quantified by total Fe, active Fe, leaf chlorophyll meter, photosynthetic parameters, enzymes, plant growth parameters, and visual ratings of leaf chlorosis. One of the distinctive characteristics of iron deficiency in field crops is the lack of correlation between leaf iron content and chlorosis. This has been termed the "chlorosis paradox." Therefore, leaf chlorophyll contents are generally used to monitor iron chlorosis [61]. The use of visual ratings and readings of a portable chlorophyll meter are the most efficient approaches to define iron chlorosis in citrus [51]. Yesiloglu et al. [40] established a screening

study for the physiological evaluation of global tolerance to lime-induced Fe chlorosis in rootstock collection of Çukurova University in the frame of the project CIBEWU, No: 015453 and evaluated by using modified "weighted-ranking" method based on the parameters of total Fe, active Fe, chlorophyll fluorescence (Fv'/Fm'), leaf chlorophyll concentration by SPAD readings, visual ratings of leaf chlorosis, and shoot and root weight.

Local trifoliate, Rubidoux trifoliate, and Benecke trifoliate exhibited severe chlorosis and were more chlorotic than other genotypes. Cleopatra mandarins among mandarin and its hybrids were more tolerant than Sunki and Calamondins. Macrophylla was the best in lemon and lemon hybrids group. Campbell [50] reported that Macrophylla was well adapted to soils of high pH. Volkameriana and Rangpur were almost the same. Gou Tou was really so tolerant to iron deficiency. All sour oranges were similar to each other. Alanya Dilimli sweet orange is a variety selected in south of Turkey. It is known to be very resistant to high pH conditions. The results confirm that Alanya Dilimli is very tolerant to high pH. According to the results, Carrizo citrange, Flhorag1, Macrophylla, Antalya Cleopatra mandarin, Tuzcu 31-31 sour orange, Gou Tou sour orange, and Alanya Dilimli sweet orange were more tolerant than others in citrus rootstock collection of Çukurova University (Table 2 – unpublished data).

Genotypes	Tolerance to iron chlorosis
Rubidoux trifoliate 08A 30.15	2
Citremon 34 12 N	3
Citrumelo 4475 SRA	3
Arec Rubidoux trifoliate	3
Arec Swingle citrumelo	2
Benecke trifoliate	2
Swingle citrumelo 4475 CRC	2
Carrizo citrange	4
Flhorag1	5
Pomeroy trifoliate SRA	2
Sacaton citrumelo I	2
Sacaton citrumelo	2
Citrumelo 1452 CRC	2
Local trifoliate	1
Troyer citrange	3
Antalya Cleopatra mandarin	4
<i>Citrus sunki</i>	4
Calamondin CRC	2

Genotypes	Tolerance to iron chlorosis
Calamondin 108 USDA	1
Tuzcu Cleopatra mandarin	2
Volkameriana CRC 01	3
Volkameriana CRC 02	3
Macrophylla	4
Rangpur lime	3
Volkameriana	3
<i>Citrus ichangensis</i> CRC	3
<i>Citrus sulcata</i>	2
<i>Severinia buxifolia</i> SRA	2
Alanya Dilimli sweet orange	5
Gou Tou sour orange SRA 506	5
Smooth Seville sour orange	3
Taiwanica	2
Tuzcu 31-31 sour orange	5
Tuzcu 891 sour orange	4

*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

Table 2. Classification of the rootstocks in collection of Çukurova University in terms of tolerance of iron deficiency.

Although trifoliolate orange has many advantages in terms of tolerance to abiotic and biotic stresses, it is susceptible to calcareous soil conditions. However, there are some superior genotypes to improve the tolerance to high pH of present rootstocks by hybridization and crossing [44].

Several rootstock breeding programs have been carried out by different countries leading in citriculture. Forner et al. [62] reported two new citrus rootstocks named F-A 5 and F-A 13, released in Spain. These rootstocks are hybrids of Cleopatra mandarin × Rubidoux trifoliolate crosses with a high level of tolerance to lime-induced iron chlorosis. Besides, Bowman and Rouse [63] mentioned a new citrus rootstock named as US-812 which is a hybrid obtained by a cross between Sunki mandarin (*Citrus reticulata*) and Benecke trifoliolate orange in USDA Indio Research Station, California. They have reported that using Valencia trees budded on to US-812 resulted in some tolerance to high alkalinity under pH conditions 8.1– 8.3. Moreover, Federici et al. [64] indicated that three citrus rootstocks released in August 2009 by the University of California named as "Bitters," "Carpenter," and "Furr" trifoliolate hybrids, tested as C22, C54, and C57, respectively, by crossing Sunki mandarin × Swingle trifoliolate orange. Bitters were found to be very tolerant to calcareous soil, whereas Carpenter and Furr were found to be moderately tolerant.

2.3. Drought

Mediterranean region has a subtropical climate and is an important region for citriculture. A significant amount of high-quality citrus fruits is produced in Mediterranean countries such as Spain, Turkey, Italy, Greece, Egypt, Morocco, and Tunisia. Fruit yield and quality are affected by genetic traits together with environmental factors. Fruit crops are frequently exposed to environmental stresses spontaneously or by conventional agronomic conditions. Some of these conditions such as high temperature may last only for a short period of time, whereas lack of water in soil may last for longer periods. Global warming is a type of greenhouse effect which is defined as the increase of Earth's average surface temperature due to the effect of greenhouse gases, such as carbon dioxide emissions from burning fossil fuels or from deforestation, which trap heat that would otherwise escape from Earth.

Yaacoubi et al. [65] indicated that Mediterranean fruit tree production is facing major changes that have environmental and socioeconomic consequences. Climatic changes related to temperature warming have been reported worldwide.

Drought stress, as one of the most ominous abiotic factors limiting the productivity of horticultural crops, is increasingly growing in dimension of severity in many regions of the world [66]. In general, the mechanism of drought resistance in plants can be explained as drought escape, drought avoidance, and drought tolerance. These traits consist of osmotic adjustments, cell membrane stability, epicuticular wax, partitioning and stem reserve mobilization, manipulation and stability of flowering processes, and seedling drought traits.

Drought tolerance is a complex trait that is important at different growth stages and involves multiple adaptations. Fundamental to this is the ability to maximize the extraction of water from the soil while minimizing loss from the leaves. Morphological adaptations include the development of deep roots and alterations in leaf morphology and cuticle structure, while physiological adaptations involve changes in stomatal density to maximize water uptake and retention [67, 68, 69].

Drought stress effects on the plant may range from slight suppression of growth and yield to temporary wilting, in which leaves flag but recover after transpiration demands decrease, to permanent wilting in which the plant suffers injury and death [70]. A plant responds to a lack of water by halting growth and reducing photosynthesis and other plant processes in order to reduce water use. As water loss progresses, leaves of some species may appear to change color, usually to blue-green. Foliage begins to wilt and, if the plant is not irrigated, leaves will fall off and the plant will eventually die. Drought lowers the water potential of a plant's root and upon extended exposure, abscisic acid is accumulated, and as a result stomatal closure occurs. This reduces a plant's leaf relative water content. The time required for drought stress to occur depends on the water-holding capacity of the soil, environmental conditions, stage of plant growth, and plant species [71]. Plants growing in sandy soils with low water-holding capacity are more susceptible to drought stress than plants growing in clay soils. A limited root system will accelerate the rate at which drought stress develops. A plant with a large mass of leaves in relation to the root system is prone to drought stress because the leaves may lose water faster than the roots can supply it. The root system has a great importance when the plant faces

drought. For instance, Rough lemon rootstocks are very drought tolerant because of their extensive, deep root systems. Newly installed plants and poorly established plants may be especially susceptible to drought stress because of the limited root system or the large mass of stems and leaves in comparison to roots.

Citrus, a perennial crop with a long orchard life, is likewise a globally important fruit crop responsible for world trade and often exposed to the vagaries of soil and atmospheric drought stress [72]. Drought stress is known to restrict the vegetative growth and yield of citrus, in addition to adversely affecting fruit quality and incurring huge economic loss to the citrus growers [73]. Therefore, screening and selection of germplasm are of great importance in terms of drought tolerance.

Pedrosoa et al. [74] reported that citrus rootstocks have differential capacities for supplying shoot tissues with water and carbon, improving the resistance to biotic and abiotic stresses and affecting plant water status and photosynthesis. Water relations have been well studied in citrus trees, showing that rootstocks alter the physiological performance under water deficit through variations in plant hydraulic conductance, leaf water potential, and stomatal conductance [75, 76, 77]. In addition, several studies have found that citrus rootstocks showed different performances when they are exposed to drought [13, 73].

Treeby et al. [78] investigated irrigation management and rootstock effects on navel orange and reported that irrigation management is far more critical for external fruit quality for trees on sweet orange and, to a lesser extent, trees on the citranges compared to trees on trifoliolate orange and Cleopatra mandarin.

Some studies also indicate that using tetraploid rootstocks increases the drought tolerance in comparison to their diploid clones in citrus. Allario et al. [79] reported that polyploidy is common in many plant species and often leads to better adaptation to adverse environmental conditions. The authors examined the drought tolerance in diploid (2x) and autotetraploid (4x) clones of Rangpur lime (*Citrus limonia*) rootstocks grafted with 2x Valencia Delta sweet orange (*Citrus sinensis*) scions, named V/2xRL and V/4xRL, respectively. The results of the authors showed that using tetraploid clones of Rangpur lime had increased the drought tolerance in grafted sweet orange.

3. Germplasm and genetic variability

The origin of citrus is believed to be southeast Asia, including south China, northeastern India, and Burma. Commercial citrus species and related genera belong to the order Geraniales, family Rutaceae, and subfamily Aurantoidea. Fruit crops is a very heterogeneous group of plants including trees, shrubs, climbing vines, and perennial herbs. They inhabit different climates, ranging from tropical to subarctic zones and altitudes from sea level to higher mountains. Fruit crops also differ considerably with respect to their origin, taxonomy, and breeding systems. The biodiversity in plants has been progressed by natural and artificial hybridization and mutation which are the basic resources of biological evolution. The deter-

oration of ecosystem directly or indirectly by human beings has always been causing the destruction of biodiversity and many advanced genotypes [80].

There is extensive genetic diversity in citrus. Aydin and Yesiloglu [81] reported that the genus *Citrus* L. belongs to the subtribe Citrinea, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family [82]. The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae, and Merrillinae, and Citreae has Triphasiinae, Citrinae, and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups: primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella, and Citrus [83].

All rootstocks and varieties used are included in the genus *Citrus*, except for kumquats (*Fortunella* spp.) and trifoliate orange (*Poncirus trifoliata* L. Raf.), the latter is used exclusively as a rootstock. Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is an important citrus relative for breeding new rootstocks. In addition to its tolerance to citrus tristeza virus, citrus nematodes, and cold weather and edaphic conditions, the dominant nature of the "trifoliate" leaves of trifoliate orange is a useful morphological marker in the visual identification of hybrids from crosses using trifoliate orange as male parent. Some hybrids of commercial interest, including citranges (sweet orange × trifoliate orange) and citrumelos (grapefruit × trifoliate orange), are used as rootstocks.

In general, the diversity of genetic structures in the subfamily of Aurantioideae, which occurred within the steps of biological evolution, is the genetic resource that has been lost before the determination of their characteristics during the rapidly vanishing process. Preservation of this kind of material is a challenge particularly for "conservation breeding" branch of plant breeding [80]. The determination of citrus genus and close relative genus and species collections with *in situ* and *ex situ* structure in the selected countries and regions, describing and establishing international legal tender for them, are very important.

The richness of germplasm has benefited genetics and breeding research in the countries that have a long history of citriculture. China is the most important place of origin for citrus. The long history and diversified climates enable China to harbor the most citrus varieties. Southern China is one of the centers of diversity for *Citrus* and related genera such as *Fortunella*. A National Citrus Germplasm Repository was established in China in the early 1980s, and a record in 1996 reported 1041 accessions [84, 85]. In India, there is an *in situ* germplasm including 627 accessions as it was reported by Singh [86], and eight *ex situ* citrus conservation collections have been established. Three *in situ* collections were established in Malaysia. In addition, there are three collections in Indonesia and Thailand, two collections in Philippines. The orchards of The Federal Fruit Crops Research Station in Tsukuba, Okitsu, and Kuchinotsu have the widest diversity collection of citrus and the relative types. It is declared that there are totally more than 1300 accessions in these three stations [84]. The original natural dispersion areas of Microcitrus and Eremocitrus as well as many relative types of citrus are located in Australia and the main collection in Australia is located at the Biological and Chemical Research Institute under the NSW Agriculture & Fisheries Depart-

ment in Rydalmer in New South Wales [84]. Tuzcu [80] reported that the collection of citrus in California began in the 1890s, and the conservation and utilization system of these were structured in 1910, shortly after the establishment of Citrus Experiment Station (CES) in Riverside [87]. H.J. Webber, the first manager of the station, ensured the establishment of Citrus Variety Collection in 1917 in the field which is currently in the Riverside Campus Area of California University. United States Department of Agriculture (USDA) National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) was established as a top organization for the coordination of citrus genetic resources studies in 1974. It is working in close cooperation with other two establishments. There are about 350 virus-free accessions under its conservation [84]. The most important collection is located at National Research Center for Cassava and Fruit Crops, CNPMF in Cruz das Almas/Bahia, which includes 1858 accessions in Brazil. Valencia Agricultural Research Institute [Instituto Valenciano de Investigaciones Agrarias (IVIA)] is responsible for all the actions regarding citrus genetic resources. According to the last records, there are 478 elite accessions in total including 13 genera (16 accessions) of the Aurantioidea subfamily in addition to Citrus genus in IVIA [84, 88]. In France, 1300 accessions at Agricultural Research Station SRA tied to National Agricultural Research Institute (Institut National de la Recherches Agronomiques – The National Agronomic Research Institute (INRA) at San Nicola in Corsica Island exist in "SRA Citrus Collection" [84]. In terms of citrus genetic resources, mainly, there is one established citrus germplasm in Turkey. This establishment is named as Tuzcu Citrus Collection (TCC) and consists of 964 accessions in Çukurova University Faculty of Agriculture [80].

4. Some developed citrus rootstocks and their tolerance to abiotic stress

The first use of rootstocks in citriculture was in 1842 against *Phytophthora* in Azores Islands through the use of resistant rootstocks. Since then, commercial citrus orchards are established by combining scion and rootstocks in order to achieve the highest quality for the scion. The use of rootstocks in citrus decreases the long juvenility period and allows the cultivation of citrus under several abiotic and biotic stress conditions. The physiology of the whole tree is affected by rootstock, including traits of economic relevance such as fruit yield, fruit size, juice quality, tree vigor, and resistance against biotic and abiotic stresses [89]. Fruit maturation, fruit holding on tree, and postharvest preservation are also affected by rootstock [90].

There is no ideal rootstock in order to manage all abiotic and biotic stress conditions. For instance, sour orange (*Citrus aurantium* L.) which has a high adaptation capability to different soil conditions has been the most commonly used rootstock in commercial citrus trees. Also, sour orange (*Citrus aurantium* L.) is tolerant to root rot, citrus blight disease, calcareous soils, water deficit, and cold, inducing high yield and high fruit quality. However, sour orange is susceptible to citrus tristeza virus, and the usage of this rootstock is decreasing in some countries due to the existence of CTV. On the contrary, Rangpur lime (*C. limonia*), Volkamer lemon (*C. volkameriana* V. Ten. and Pasq.), and rough lemon (*C. jambhiri* Lush.) are drought resistant and increase the fruit yield of the grafted scion. Besides, they reduce the fruit quality

as compared to fruits obtained from sour orange, trifoliolate orange, Carrizo citrange, and Troyer citrange. However, they are sensitive to cold.

The search for new rootstock in citrus production as well as many fruit species is necessary to sustain production under the inevitable abiotic stresses in many different ecological conditions of the citrus-growing areas of the world. Also, new diseases, the spread of the known diseases, and different environmental conditions affected by the climatic change force the demand for developing new citrus rootstocks. Below, we have tried to cover and explain some of the newly released citrus rootstocks obtained by breeding programs carried out in several countries by several researchers.

Swingle citrumelo is a hybrid of Duncan grapefruit and trifoliolate orange produced in 1907 by Swingle and released by the United States Department of Agriculture in 1974. Since then, it has been used successfully as a rootstock in many countries. Most varieties produce very satisfactory yields of good to excellent quality fruit on Swingle citrumelo. Fruits produced on Swingle citrumelo are similar to sour orange, Carrizo, and Troyer citranges in terms of fruit quality. The trees have good cold-hardiness only slightly inferior to that of trees on trifoliolate oranges. Scions on Swingle citrumelo are very tolerant to CTV, blight, and root rot as well as being resistant to citrus nematodes. They also have good exocortis and xyloporosis tolerance. The trees grow well on most soils and are reportedly especially tolerant of waterlogged conditions. In contrast, Swingle citrumelo is an unacceptable choice in heavy clay, calcareous soils, and high pH soils. Swingle is sensitive to high pH soils and is unsuitable for highly calcareous soils. Therefore, it is not so popular in many Mediterranean countries. Tolerant to environmental and soil conditions, Swingle citrumelo is sensitive to high chloride levels in soil and irrigation water but is more salt tolerant than other trifoliolate hybrids such as Carrizo and Troyer citranges. Swingle has moderate drought tolerance [91].

Citranges are known as hybrids of sweet orange and trifoliolate orange. The main purpose of the citrange development in Florida was to combine good traits of sweet orange with the cold-hardiness of the trifoliolate orange in order to create cold hardy scions. Although unsuccessful, a most significant source of new rootstocks was produced instead. There are several named selections, the more important of which are Carrizo and Troyer citranges. These are hybrids of Washington navel orange and *Poncirus trifoliata*. The original crosses were made in the early 1900s by the United States Department of Agriculture with the intention of producing cold-tolerant scion varieties. They were later identified as being suitable for use as rootstocks. Fruit quality of the scion on Carrizo and Troyer is excellent. Trees on both grow moderately vigorous on a range of soil types but have poor salt tolerance and are sensitive to calcareous soils and exocortis virus. They have intermediate frost tolerance but are less cold-hardy than those on Cleopatra mandarin and trifoliolate orange. In Turkey, Troyer citrange and trifoliolate orange show superior fruit quality for Satsuma mandarins, and they are the mainly used rootstock in Aegean region of Turkey. However, using Carrizo citrange as a rootstock to especially mandarins and oranges is more common due to high soil pH levels in the Mediterranean region of Turkey. In general, the performance of Carrizo citrange is slightly better than Troyer in cancerous soils of Çukurova. However, sour orange is the main rootstock in Turkey. Ninety percent of citrus varieties in Turkey are grafted on the sour orange rootstock.

Benton citrange is a hybrid of Ruby Blood orange and trifoliate orange. It was bred in the late 1940s by the Department of Agriculture, New South Wales, Australia. The seed was first released to the industry in 1984. There are some commercial plantings using this rootstock which were established in 1990, but poor seed production in the seed source trees has been an impediment to its widespread usage. Because of its erratic performance under orange and mandarin scions, it is only recommended for Eureka lemons in Australia and only replant situations in Queensland. In Florida, Benton citrange is recommended for small-scale commercial trials with oranges and grapefruit. Trees on Benton rootstock are reported to be moderately cold tolerant and higher yielding.

C-35 citrange was bred by the University of California and released in 1987 and is a hybrid obtained by crossing Ruby Blood orange × Webber-Fawcett trifoliate. C-35 is tolerant to *Phytophthora* and CTV and resistant to citrus nematodes. Frost tolerance is good as or slightly better than Carrizo. Trees grow 25% smaller than Carrizo, making C-35 a candidate for closer spacing plantings. Trees grown in sandy, loam, and clay soils are satisfactory, but they are more sensitive to calcareous soils than Carrizo. C-32 has the same parentage as C-35 citrange and is a hybrid between Ruby orange and Webber-Fawcett trifoliate. Its very low seed production makes this citrange's seedling propagation difficult in order to use as rootstock [91].

Several rootstock breeding programs have been carried out by different leading countries in citrus industry in order to handle increasing problematic issues by abiotic and biotic stress factors.

Forner et al. [62] reported two new rootstocks released in Spain. Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13) are two interspecific hybrids obtained through traditional hybridization by a senior author in a program for breeding citrus rootstocks at the IVIA in Moncada (Valencia), Spain. The researchers aimed to obtain new rootstocks tolerant to CTV, salinity, and lime-induced chlorosis and resistant to *Phytophthora*. They reported the resistance of F-A 5 and F-A 13 to CTV. In addition, F-A 5 was found to be more tolerant to lime-induced chlorosis than Carrizo citrange, whereas F-A 13 is less tolerant [92]. Besides, both rootstocks have good tolerance to salinity and an excellent tolerance to flooding, as reported. Gonzalez-Mas et al. [53] conducted a rootstock field study in order to investigate rootstock effects on leaf photosynthesis in "Navelina" trees grown in calcareous soil. Authors have used seven new citrus rootstocks with Carrizo citrange obtained by J. Forner at the IVIA: F-A 5, F-A 13, F-A 418, F-A 517, 030116 (Cleopatra mandarin × *P. trifoliata*), 020324 (Troyer citrange × Cleopatra mandarin), and 230164 (*C. volkameriana* Ten. and Pasq. × *P. trifoliata*). Trees grafted on F-A 5 performed best under these calcareous soil conditions, whereas those on Carrizo citrange were poorly adapted, regarding the parameters investigated.

Bowman and Rouse [63] reported the release of US-812 citrus rootstock in May 2001 by the Agricultural Research Service of the USDA and is the result of a cross between Sunki mandarin and Benecke trifoliate. The rootstock was found to be highly productive of good quality fruit with a moderate vigor (standard medium tree size) as it was reported. The US-812 shows tolerance or resistance to CTV and citrus blight. It was reported that US-812 has good soil adaptability and disease resistance. Valencia orange grafted on US-812 performed well under high pH conditions in calcareous soils. Bowman [93] also introduced US-802 and US-897 and

reported high productive per tree size, good soil adaptability and disease resistance, tolerance of Diaprepes and Phytophthora complex, large contrast in vigor, and tree size as the forthcoming features of the rootstocks. Besides, US-942 rootstock is very highly productive with good fruit quality and soil adaptability.

According to the report of Federici et al. [64], three new citrus rootstocks were released in 2009 by the University of California, Riverside. These three rootstocks were named as "Bitters," "Carpenter," and "Furr" trifoliate hybrids, tested as C22, C54, and C57, respectively, and obtained by sexual hybridization of Sunki mandarin × Swingle trifoliate orange. The main character of these three hybrids is they all show good tolerance to citrus tristeza virus.

As reported by Federici et al. [64], "Bitters" showed good tolerance to freezing. It is tolerant to CTV, moderately tolerant to *Phytophthora parasitica*, not very tolerant of citrus nematode, and very tolerant of calcareous soil. "Carpenter" showed moderate tolerance to freezing. It is tolerant to CTV, moderately tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil. "Furr" also showed good tolerance to freezing, and it is tolerant to CTV, very tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil [64].

5. Breeding techniques for tolerance to abiotic stress: Traditional and biotechnological approaches

Nearly all commercial citrus in the world are grown as grafted trees, with the scion cultivar budded on a selected rootstock cultivar. A good scion and rootstock combination supports the development of trees that bear large quantities of high-quality fruit. However, many available rootstocks are inadequate to meet the emerging needs and challenges. A large proportion of the problems faced by the citrus industry could be overcome by the use of improved rootstocks [93, 94]. However, developing an improved rootstock is a long-term approach because of the several difficulties, mainly the complexity of citrus biology. Typically, it takes at least 15 years from the beginning of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use.

5.1. Traditional breeding

Although somatic hybridizations via *in vitro* culture methods and genetic transformation via the regeneration process of plant tissues have opened new enthusiastic prospects for citrus genetic improvement, classical breeding techniques still remain important for citrus breeding. Genetic variations have a great importance in terms of plant breeding. These variations can be obtained either spontaneously or artificially by mutations and sexual hybridizations in order to achieve specific breeding objectives. Planned or unplanned sexual hybridizations have been responsible for the evolution of the new genotypes for using either rootstock or scion. Many intergenic hybrids were produced by controlled pollination. For example, citranges (*C. sinensis* × *P. trifoliata*), citrumelos (*C. paradisi* × *P. trifoliata*), citremons (*C. lemon* × *P. trifoliata*),

citradia (*C. aurantium* × *P. trifoliate*), citrumquat (*Fortunella* spp. × *P. trifoliate*), and Eremoradia (*Eremocitrus glauca* × *C. aurantium*).

Citrus flowers usually bloom in the spring in one great flush, except acid limes and lemons which are noted for flowering throughout the year in cold subtropical climates. In tropics, flowering may occur more than once throughout the year. In addition, drought and excessive fertilizing may induce flowering. Citrus flowers are mostly hermaphrodite and release pollen when the stigma is receptive. However, there are some exceptions such as staminate and pistillate flowers occurring in lemons Satsumas, Shamouti, and sour orange. Besides, late harvest of the fruits instead of optimum harvest time and fertilization deficiencies can increase the ratio of staminate and pistillate flowers on trees. Besides, W. Navels are known to have the ability to set parthenocarpic fruits due to their pollenless flowers. The flowers never close; the petals merely shed a few days later. The stigma becomes receptive just before the bud breaks open, but the stamens usually do not release pollen until several hours later, after the flower is fully open. This should be considered in terms of collecting flowers for pollens to be used as male parents. Most pollination in citrus is done by insects except for varieties showing parthenocarpy that no pollination is required for fruit development.

Many citrus cultivars are known to be self-incompatible and, in some cases, cross-incompatible. With such cultivars, an appropriate pollen supply and pollinating agents is needed. Pollination requirements vary among the species and cultivars. For example, open pollinated flowers of grapefruit result in significant increases in both fruit number and seed numbers. When lemons are protected from insect visitations, a set of fruits decreases. Pummelos are known to be self-incompatible as well as Clementines, Lee, Page, Nova, and Robinson. In contrast, no pollination problems have been observed in citron, kumquat, Meyer lemon, and trifoliate orange, but there have been problems of seed set in "Morton" and "Troyer" citrange [95].

We consider the apomixes as one of the major problems in citrus rootstock breeding. Nucellar embryony is the most unusual feature that exists in the reproductive biology of citrus. This mechanism limits crossing and selfing in many varieties. Most of the genotypes that can play important roles as female parents in traditional crossing studies are highly apomictic (for example, see Table 3, unpublished data recorded at the Çukurova University, Faculty of Agriculture, Department of Horticulture, Citrus Germplasm Orchards). Hence, citrus breeding is limited by nucellar embryony of most diploid genotypes [96, 97]. The nucellar tissue which surrounds the megagametophyte can produce additional embryos (polyembryony) which are genetically identical to the parent plant. In contrast, zygotic seedlings are sexually produced and inherit genetic material from both parents. Zygotic and nucellar embryos can occur in the same seed. Not all citrus species exhibit the characteristic of polyembryony, some produce only zygotic embryos (pummelo, citron, Clementine, Temple, and Persian lime, for example). Others produce only nucellar embryos. Many citron and lime varieties produce a significant percentage of zygotic seedlings but oranges, grapefruit, and many mandarins usually have a low percentage.

Genotype	Polyembryony (%)
Tuzcu 01-21 sour orange	60
Carrizo citrange	92
Troyer citrange	71
Swingle citrumelo	50
Local trifoliolate (<i>P. trifoliata</i>)	80
Cleopatra mandarin	95
Volkameriana	60
Rough lemon	56
Florida Rough lemon	93
Sunki mandarin	50
Yuzu	60
Taiwanica	50
King mandarin	81
<i>C. myrtifolia</i>	85
<i>C. obovidea</i>	76

Table 3. Polyembryony ratio of some potential genotypes in CU Citrus Germplasm Orchards to be used in breeding studies.

In addition, sexual hybridization faces some constraints in citrus, due to high heterozygosity, long juvenility, and polyembryony of most citrus cultivars. Moreover, it is difficult to identify sexual hybrid embryos in their early stage. In this case, using trifoliolate oranges, which are valuable rootstocks due to their characteristics such as cold-hardiness and resistance to root rot, CTV, and nematodes, gains another importance for citrus rootstock breeding against polyembryony. Since the trifoliolate character is dominant, progenies exhibiting the trifoliolate phenotype of the pollen parent can be considered as putative hybrids. So using trifoliolate trait as a morphological marker is useful for early separation and characterization in citrus rootstock breeding studies. In contrast, progenies obtained by crossing combinations using polyembryonic genotypes as females have to be identified via molecular marker systems (RAPD and SSR) in order to speed up the separation of zygotic hybrids from nucellar seedlings.

Controlled cross-pollination in citrus is mainly performed for combining desirable traits from different genotypes or species and inducing heterosis. Based on this method, many hybrid rootstocks between citrus and *Poncirus* have been developed (see Section 4).

Controlled pollination is relatively easy in citrus. Seed parent and pollen parent flowers should be protected against contamination. Emasculation is generally easy and less effortless at the flowers that are nearly ready to open. Emasculation is accomplished by gently separating the petals, pulling off the anthers while avoiding contact with stigma. Pollination should be carried

out immediately after emasculation. A special storage of the pollen is seldom necessary while crossing within the genus *Citrus*. The genera *Poncirus* and *Fortunella* can be crossed with *Citrus*. Trifoliate orange naturally bloom earlier than citrus, so the pollens must be stored until the flowering time of *Citrus*. Pollens should be collected from unopened flowers from the branches of trees. After a waiting period of 24 h in the room temperature, a high quantity of pollen grains can be collected from anthers. Calcium chloride can be used as a drying agent just before storage of the pollen grains in a cold condition. On the contrary, *Fortunella* bloom much later than citrus in many areas. Figure 5 presents a traditional breeding procedure in citrus at Çukurova University, Citrus Germplasm Orchards.

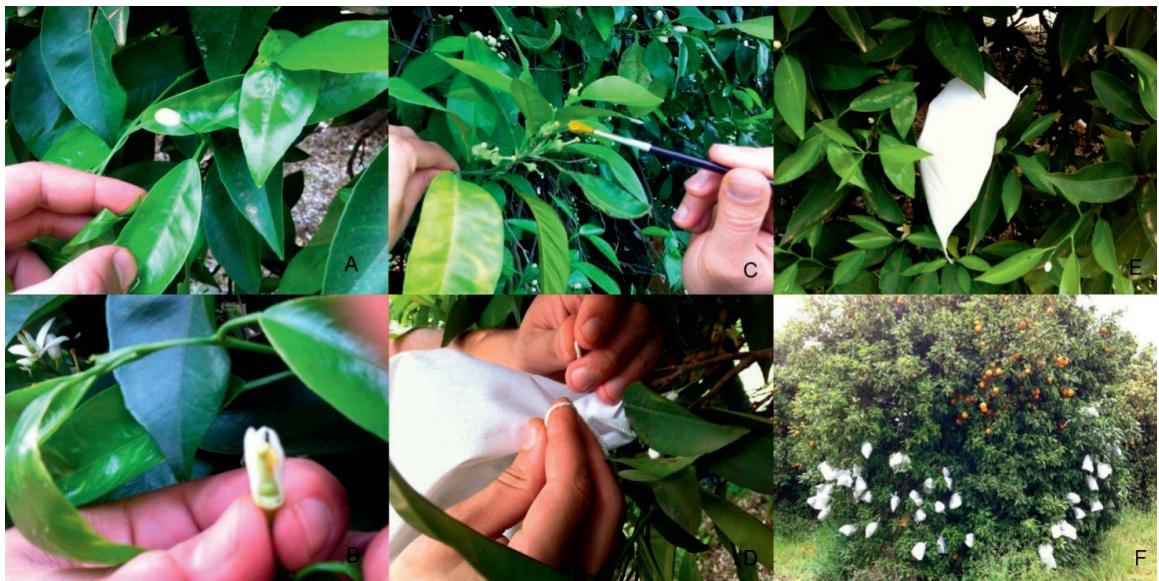


Figure 5. Traditional cross-hybridization in citrus. (A) a large unopened bud, (B) emasculation, (C) pollination of the emasculated flower, (D) cotton pad wrapping around the twig, (E) bagged twig, (F) general view of the seed parent after crossing.

Fruit breeding, especially using classical breeding methods, is a difficult work taking a lot of time. In terms of citrus, chance seedlings were the main source for the cultivars, and spontaneous mutations on branches were used to select new cultivars. Current breeding projects in the present day is crossing superior selections and inducing mutations for seedlessness as well as crossing at different ploidy levels for seedless triploids.

5.2. Current biotechnologies applied in rootstock breeding

Genetic improvement of citrus through conventional breeding is limited by their genetic and reproductive characteristics. Citrus species have a complex reproductive biology, with many cases of cross- and self-incompatibility, apomixis, and high heterozygosity, and most of them have very long juvenile periods. Most species are highly heterozygous and produce progeny segregating widely many characters when crosses are made. In addition, juvenile periods are often extensive and most significantly, the presence of adventitious embryos in the nucellus of developing ovules of most citrus types greatly inhibits hybrid production [97, 98].

Plant somatic hybridization via protoplast fusion has become an important tool in plant improvement, allowing researchers to combine somatic cells (whole or partial) from different cultivars, species, or genera resulting in novel genetic combinations including symmetric allotetraploid somatic hybrids, asymmetric somatic hybrids, or somatic cybrids [99].

Briefly, the development of hybrid plants through the fusion of somatic protoplast derived from different sources of two different plant cultivars, species, and genera is called somatic hybridization. The technique of somatic hybridization involves the following steps: (1) isolation of protoplasts, (2) fusion of the protoplasts obtained from desired genotype, (3) culturing the hybrid cells, and (4) regeneration of hybrid plant. Mechanical or enzymatic methods can be used for the separation of protoplasts from plant tissue. However, the mechanical method is a laborious process that has some disadvantages such as low yield of protoplast and low protoplast viability. A plant cell consists of cell wall which has to be degraded if the protoplasts of the cell have to be manipulated as required. For this purpose, the plant cell is treated with enzymes, such as pectinase, macerozyme, cellulase, etc., that hydrolyze the plant cell wall. Since protoplasts are present in every plant cell, it can be theoretically isolated from all parts of the plant. But most successful isolations are made possible from the leaf of the plants.

Once purified protoplasts have been obtained from two different plant or tissue sources, various treatments can be given to induce them to fuse together. Generally, chemical agents or electrical manipulation is necessary to induce membrane instability that leads to protoplast fusion.

Polyethylene glycol (PEG) is used most frequently in conjunction with alkaline pH and high calcium concentrations. There are a number of steps in the fusion of plant protoplasts using PEG as a chemical facilitator. Another type of cell fusion that has emerged in recent years involves the manipulation of cell membranes by electrical currents. This process involves passing low-voltage electric pulses in a solution of protoplasts to be fused so that they line up for fusion. The protoplasts can be fused by subjecting it to brief exposure to high-voltage electric current which leads to alteration of membrane so that the adjacent protoplasts fuse. Electrofusion of plant protoplasts is often preferred over PEG fusion because it does not employ reagents that are toxic to the cells being fused. As with all other procedures, the conditions for electrofusion must be optimized for specific cell types to achieve maximum effectiveness. Typically, a yield of 20% or greater fusion products can be obtained by electro-fusion of protoplasts compared to less than 1% fusion products with PEG [100].

Somatic cell fusion could overcome sexual incompatibility and long juvenility and may play a potential role in citrus genetic improvement, including producing directly or indirectly superior varieties, improving citrus scion and rootstock, or creating allopolyploids for triploid breeding [101, 102]. As Grosser and Gmitter [103] reported, this technique can facilitate conventional breeding, gene transfer, and cultivar development by bypassing some problems associated with the conventional sexual hybridization including sexual incompatibility, nucellar embryogenesis, and male or female sterility. Conversely, somatic hybridization is very promising for citrus rootstock breeding for combining genotypes having different tolerance to abiotic stress [104]. Citrus rootstock differs in terms of tolerance/resistance to abiotic stress

conditions. Incompatibility between some genotypes that have high level of tolerance to abiotic stress conditions limits the usage of traditional hybridization [105]. Oigawara et al. [106] reported the first intergeneric citrus hybrids obtained by combining embryonic callus of sweet orange and *Poncirus trifoliolate* leaves via protoplast fusion. Grosser et al. [107] indicated that the regeneration of more than 300 plants obtained by protoplast fusion of Hamlin sweet orange and Flying Dragon trifoliolate. The regenerated plants were determined as tetraploids. Kobayashi and Ohgawara [108] recovered tetraploid somatic hybrids by fusing the protoplast obtained from the embryonic callus of Trovita orange and leaf mesophyll protoplasts of Troyer citrange. Grosser et al. [109] reported tetraploid somatic hybrids obtained by fusing the protoplast via PEG method. They used several manipulations such as Cleopatra mandarin (*Citrus reshni*) + trifoliolate orange (*Poncirus trifoliata* (L) Raf.), Acidless orange (*Citrus sinensis* (L) Osb.) + trifoliolate orange (*Poncirus trifoliata* (L) Raf.), sour orange (*Citrus aurantium* L) + Flying Dragon trifoliolate (*Poncirus trifoliata*), sour orange (*Citrus aurantium* L) + Rangpur lime (*Citrus limonia* Osb.), and Milam lemon + Sun Chu Sha mandarin (*Citrus reticulata* Blanco). Tetraploid plants were identified and propagated for further rootstock experiments. Ollitrault et al. [104] had reported the first intergeneric somatic hybrid obtained from protoplast fusion between *Citrus reticulata* + *Fortunella japonica* in France. The authors have regenerated approximately 100 plantlets by several manipulations (*C. reticulata* + *C. sinensis*, *C. reticulata* + *C. paradisi*, *C. reticulata* + *C. limon*, *C. reticulata* + *C. aurantifolia*, *C. reticulata* + *Poncirus trifoliata*, and *Citrus aurantium* + *Eremocitrus glauca*) and reported the possible use of these population as parental germplasm for both scion and rootstock breeding programs in citrus. Grosser et al. [99] indicated that the somatic hybrids obtained by *Citrus* + *Severinia* and *Citrus* + *Fortunella crassifolia* had lower performance as rootstocks, whereas promising performance was recorded from the scion grafted on somatic hybrids obtained from the manipulations of Acidless orange + *Atalantia ceylanica* and Nova mandarin + *Citropsis gilletiana*. Also, the researchers reported the dwarfing effects of somatic hybrids obtained by fusing the protoplast of sour orange + Flying Dragon and Cleopatra mandarin + Flying Dragon. Ollitrault et al. [16] had selected 11 allotetraploid somatic hybrids by using flow cytometry and molecular markers and propagated them for rootstock trials in order to investigate their tolerance to abiotic and biotic stress. Mourão Filho et al. [110] reported the root rot tolerance of the somatic hybrids ("Cleopatra" mandarin + "Volkamer" lemon, "Cleopatra" mandarin + sour orange, "Caipira" sweet orange + "Volkamer" lemon, and "Caipira" sweet orange + "Rangpur" lime). Somatic hybrid combinations involving sour orange or *Fortunella obovata* as one of the progenitors were intolerant to CTV. They suggested future field evaluations with somatic hybrids, especially those with tolerance to CTV.

In addition to these findings, tetraploid rootstocks usually have a built-in tree-size control mechanism due to some unknown physiological reaction with the diploid scion. Mourão Filho et al. [110] indicated that plants budded on tetraploid rootstocks are generally smaller, which could lead to reduced harvest costs and greater production efficiency. In Florida, more than 70 somatic hybrids that can potentially be used as rootstocks have already entered into commercial field trials. Preliminary results from these trials have shown that somatic hybrid rootstocks can produce adequate yields of high-quality sweet oranges (*Citrus sinensis* L. Osbeck) on small trees [111]. Ollitrault et al. [112] reported an intergeneric somatic hybrid

between Willow leaf mandarin and Pomeroy trifoliolate named as "Flhorag1." Dambier et al. [44] reported the agronomic evaluation of the Flhorag1 in Morocco. Valencia orange trees on Flhorag1 displayed the lowest growth followed by Carrizo citrange and Volkamer lemon in an agreement regarding tetraploid rootstocks controlling the tree size [109]. Flhorag1 also proved highly tolerant to iron deficiency (unpublished data of Çukurova University obtained within the framework of the INCO "CIBEWU" project).

Genetic transformation is also an attractive alternative technique for citrus genetic improvement. Almeida et al. reported that genetic transformation in Citrus has been obtained mainly from juvenile material such as embryogenic cells, epicotyl segments from *in vitro* germinated seedlings, and internodal segments from plants cultivated in the greenhouse due to a higher morphogenic ability compared to that of mature tissues [113, 114, 115, 116]. Peña et al. [18] concluded that the transformation efficiencies are generally low, and protocols are dependent on species, or even cultivar dependent. One of the limitations within this technology is low plant regeneration frequencies especially for many of the economically important citrus species [117, 118].

Another big area of biotechnology is DNA marker technology, derived from research in molecular genetics and genomics, which offers great promise for plant breeding. Owing to genetic linkage, DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased. The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of "molecular breeding" [119].

Genomic research in recent years led to the development of screening tools via marker-assisted selection, which enables much more efficient selection of superior recombinants improved for multiple traits from conventional breeding efforts. MAS can increase the efficiency of citrus breeding and may speed the release of new cultivars. In this section, the possibilities of using MAS method for early selection in citrus rootstock breeding programs will be discussed.

MAS can be very useful to efficiently select for traits that are difficult or expensive to measure, exhibit low heritability, and are expressed late in development. However, it is usually essential to confirm at certain points in the breeding process that the selected individuals or their progeny do in fact express the desired phenotype or trait. Marker types can be classified as morphological, biochemical, cytological, and DNA based (molecular). The successful application of MAS relies on the tight association between the marker and the major gene or quantitative trait locus (QTL) analysis responsible for the trait [120].

Carillo et al. [121] reported that many studies have focused on mapping QTLs for salt tolerance-related traits in rice because of its requirement for irrigation for maximum yield, its sensitivity to salinity, and its relatively small genome. Gmitter et al. [122] reported that a localized genetic linkage map of the region surrounding the citrus tristeza virus resistance gene was developed from *P. trifoliolate*. The authors indicated that the identification of markers tightly linked to CTV will enable citrus breeders to identify plants likely to be CTV resistant by indirect, marker-assisted selection, rather than by labor-intensive direct challenge with the pathogen. For early

selection in rootstock breeding program in citrus, Xu et al. [123] suggested that the feasible application of MAS in citrus rootstock breeding for citrus nematode resistance needs at least two genetic markers, each corresponding to related locus, in order to pyramiding the multi-genes associated or cofunctioned in controlling the citrus nematode resistance.

6. Conclusions

Citrus species are the most produced fruit crops and one of the most imported fruit groups subjected in both domestic and export markets. Citrus fruits can be produced in the tropical, semi-tropical, and subtropical climates with such a great market value. Factors limiting citrus growth in these climates significantly vary. Epidemic levels of disease and pest regarding the ecological conditions (temperature, relative humidity, and solar radiation) also remarkably differ. On the contrary, the existence of common abiotic stresses such as drought, salinity, and iron chlorosis in citrus-producing countries differs depending on the countries and the production areas of the countries. Rootstock is used for a successful citrus production against the abiotic stresses discussed.

In addition, searching for dwarfing rootstock, which enables high-density planting, has gained importance in terms of increasing the fruit yield per area, fruit quality, use of mechanical pruning, harvesting and decreasing pest, and disease management in citriculture. With this knowledge, we can say that there is no ideal rootstock which can combine all these traits. Hence, countries with different ecological conditions have to manage individual rootstock programs. In contrast, abiotic and biotic factors as well as the producer demands change depending on market conditions and new trends. Thus, breeding studies have to be persistent and managed by the current production situation and future demands.

In several breeding programs, existing rootstocks or genotypes that can be used as rootstocks in citrus production have been screened for abiotic stress conditions, and their current tolerance/resistance levels had been reported. These rootstocks currently have been used in citriculture depending on the ecological conditions of the producer countries. In addition, traditional breeding studies were carried out with these genotypes in order to combine their different tolerance/resistance characteristics in one genotype. Several successful rootstocks have been obtained so far by breeding programs. But traditional breeding takes at least 15 years from the beginning of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use and limited by the complex reproductive biology of citrus. Therefore, rootstock breeders have begun to benefit using biotechnological methods in citrus breeding such as somatic hybridization, genetic transformation, and marker-assisted selection in recent years.

Genetic capacities of the parents or genotypes have great importance in breeding studies whichever method (traditional or biotechnological) is used, since the aim of breeding is to expose or combine the existing genetic capacity of parents. Therefore, screening both *ex situ* and *in situ* germplasm in the world regarding problems of citrus producer is necessary. Using promising genotypes obtained in screening studies carried out in germplasms in addition to

current citrus rootstock will result in an increase in breeding programs. As mentioned before, stress conditions are not stable and differ regarding the ecological conditions. Therefore, the sustainability of germplasm is necessary as long as human beings and citriculture exist.

Practical experience shows that abiotic stresses occur at high or low intensity in about all citricultural growing areas around the world. In this study, we have tried to explain the current situation of using citrus rootstocks for tolerance to abiotic stresses and breeding studies resulted and ongoing against abiotic stress in citrus production.

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The Transcriptional Modulation of Inositol and Raffinose Family Oligosaccharides Pathways in Plants – An (A)Biotic Stress Perspective

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Abstract

Among the multifunctional molecules that participate in processes of plant tolerance/resistance to stresses, inositol (Ins) and its derivatives (phosphorylated, methylated, oxygenated, and Raffinose Family Oligosaccharides) have attracted the attention of researchers. These compounds represent versatile and dynamic signaling molecules and osmolytes in all eukaryotes. Due to the impacts related to Ins and its derivatives in a plant cell, assays have been conducted to understand how these biomolecules affect plant physiology. Thus, overexpression or knockout of Ins-related genes has been shown as interesting strategies for generating more efficient plants capable of growing under stress conditions. In this chapter, studies using molecular tools are presented, and the impacts of their results are discussed based on the plant stress tolerance/resistance. Furthermore, an informative panel is provided with transcriptional modulation of genes related to Ins and its derivatives expressed in plants under stress. There is a gap involving about two dozen enzymes associated with the synthesis of Ins-related compounds that have not been adequately studied, and they represent an area of high biotechnological potential.

Keywords: Transgeny, tolerance, resistance, biotechnology

1. Introduction

To survive and integrate in the niche in which they germinate, plants constantly regulate their internal environment to external fluctuations encompassing soil, climate, and biological interactions. Thus, along its evolutionary processes, plants were selected through the need of

molecular mechanisms for physiological adjustments to inadequate conditions for development, resulting from adverse conditions. In this way, plants have a diverse and active cellular machinery at different stratified levels, covering perception, signaling, transcriptional control of key metabolic pathways and synthesis of molecules responsive to stresses [1].

Among the molecules functioning in more than one of the aforementioned levels, inositol (Ins; C₆H₁₂O₆) is a biomolecule of great interest. It is a cyclic carbohydrate (polyalcohol) that anchors in each of the six carbons forming the ring, a hydroxyl group. Along with their derivatives, Ins has multiple effects on plant metabolism. They act from the production of secondary messengers to the synthesis of osmolytes and antioxidants (more details in the reviews of [2, 3]). Phosphorylated Ins-derivatives [(poly)phosphoinositides and inositol (poly)phosphates] are versatile and dynamic signaling molecules in all eukaryotes, particularly in plants [4]. These two classes of compounds [highlighted in red and orange respectively, in Figure 1] are interdependent. While (poly)phosphoinositides are used in the synthesis of inositol (poly)phosphates through the action of phospholipases; the breaking of inositol (poly)phosphates produces inositol, which is a substrate for the synthesis of (poly)phosphoinositides. Moreover, according to Ins metabolism, shown in Figure 1, another branch realizes the synthesis of methylated derivatives (highlighted in green). These compounds act as important osmoregulators during periods of unfavorable conditions [5]. Additionally, oxygenated Ins-derivatives are observed (highlighted in yellow in Figure 1), which are involved in increasing plant tolerance to stresses by decreasing oxidative damage [6]. Still associated with Ins is the metabolism of the Raffinose Family Oligosaccharides (RFOs) [7]. In this biosynthetic pathway, the galactinol synthase (GolS; EC 2.4.1.123) uses myo-inositol and UDP-galactose to produce galactinol, which serve as galactose donors for subsequent synthesis of RFO members ([8, 9]; highlighted in red in Figure 2). Recent reports indicate that RFOs may assist in the reactive oxygen species (ROS) cleaning process. In periods of stress, ROS accumulation favors the physiological imbalance of plants [10].

Due to the impacts related to the Ins and its derivatives in a plant cell, assays have been conducted to understand how these biomolecules affect the physiology of plants. Thus, overexpression or knockout of genes present in these pathways has been shown as interesting strategy for generating more efficient plants capable of growing under abiotic stress conditions. In this chapter, studies using molecular genetic tools will be presented, which affect the above-mentioned metabolic pathways and the studied organisms.

2. The use of Ins and phosphorylated Ins-derivatives in plant cells under stress

Although there are several articles addressing the involvement of Ins and its derivatives in plant stress responses, so far no report has described the overall transcriptional orchestration of these components covering the metabolic pathways related to them. Information is available only in particular assays covering few genes and their expression modulations, some individual gene knockout analyses or a specific GMO (genetically modified organism) assay. In most

cases, Ins and its phosphorylated derivatives have positive impact in plant tolerance/resistance to several stresses, promoting a biotechnological interest in these compounds.

Among the Ins-derivatives, those that are phosphorylated [(poly)phosphoinositides (highlighted in red in Figure 1) and inositol (poly)phosphates (highlighted in orange)] are the most discussed in the literature. This fact reflects the importance of these compounds in plant physiology in signaling activities. An example is a work developed by Hunt et al. [11] with transgenic tobacco plants (*Nicotiana tabacum* cv. Wisconsin 38) expressing reduced levels of a gene encoding phospholipase C (PI-PLC; EC 3.1.4.11). This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to InsP₃ [inositol (1,4,5)-trisphosphate; (Figure 1)], affecting from this point on the rest of the pathway. The obtained transgenic plants showed a partial inhibition of stomatal opening due to the action of ABA (abscisic acid phytohormone). A possible role of PI-PLC enzyme and InsP₃ in ABA-dependent signaling pathway was also reported, thus suggesting that a complete response to ABA phytohormone in guard cells requires PI-PLC. However, other calcium-mobilizing pathways could also help in ABA guard cell signaling. The stomatal movement is a critical process for the plant under drought conditions by enabling better use of its water supply.

Mills et al. [12] provide more details on this ABA-mediated stomatal regulation in transgenic plants obtained by Hunt et al. [11]. A three-day assay under drought and in dark-adapted conditions, to reopen the stomata in response to light, was carried out. The results showed that transgenic plants with reduced PI-PLC as compared to control plants (with the empty vector without the transgene insert) have a greater increase in stomatal conductance. Thus, there is a strengthening of the role of inhibition due to PI-PLC in ABA-mediated stomatal opening. Further analysis indicated tobacco PI-PLC acting on the inhibition of stomatal opening by ABA, but not in promoting ABA-induced stomatal closure.

There are also reports of the involvement of Ins-derivatives in ABA-independent mechanisms during periods of drought. Perera et al. [13] obtained *A. thaliana* plants transformed with human type I gene for inositol polyphosphate 5-phosphatase (InsP₅-ptase; EC 3.1.3.56; Figure 1). This enzyme hydrolyses InsP₃, which is an essential element of the signal transduction pathway in general response to stresses. Looking at the impact on plant response to drought, the authors observed that transgenic plants showed increased stress tolerance after 12 days of watering suspension. After this period of stress, wild and controls plants (with the empty vector without the transgene insert) became brown and dry, while InsP₅-ptase transgenic lines remained green and turgid. Furthermore, transgenic plants under drought conditions showed reduced levels of ABA compared with wild plants in the same condition as well as no induction of several genes regulated by the phytohormone. The analyses of stomatal responses in transgenic plants observed that guard cells are less responsive to the inhibition of opening stomata promoted by ABA. Nevertheless, there is an increase in sensitivity to the closing of the stomata, induced by the phytohormone. The transgenic plants showed irregular behavior in coordinated processes via ABA. Despite this, these plants showed a compensatory overexpression of an ABA-independent pathway involving the transcription factor (TF) DREB2A (dehydration-responsive element-binding protein 2A) and a subset of genes regulated by this TF. In this way, the drought tolerance of InsP₅-ptase plants was mediated in part via DREB2A-

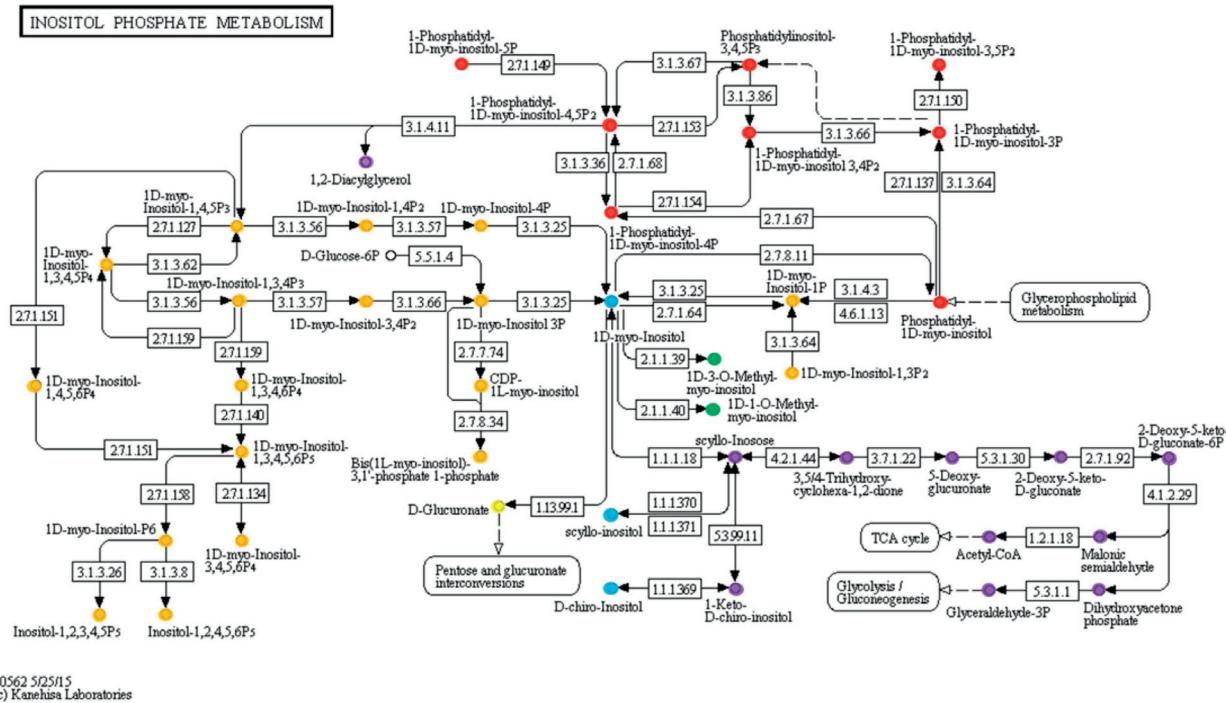


Figure 1. Inositol phosphate metabolism from the KEGG Pathway database. The stereoisomers (highlighted in blue); the phosphorylated derivatives [inositol (poly)phosphates (highlighted in orange) and (poly)phosphoinositides (highlighted in red)]; the methylated derivatives (highlighted in green); the oxygenated derivatives (highlighted in green); and others (highlighted in purple).

dependent and that constitutive dampening of InsP₃ signal revealed unforeseen interconnections between signaling pathways.

In a similar assay, Khodakovskaya et al. [14] reported physiological consequences in transgenic lines of *Solanum lycopersicum* (cv. Micro-Tom) overexpressing a human type I InsP5-ptase gene. The transgenic lines presented a content of 15–30% of InsP3 observed in the wild-type plants. This reduction led to increases in: (1) the total vegetative biomass (two- to fourfold) with an increased ratio of root:shoot dry weight; (2) lycopene levels (in fruit); and (3) the hexose concentration (on fruits and leaves). After 13 days of water stress, the leaf water potential in transgenic plants was about -0.4 MPa higher than in control plants. Drought tolerance in transgenic lines was associated with increased hexoses in the leaves. This would contribute to maintaining a greater potential for water in transgenic leaves under drought. Furthermore, increases in the number of root biomass may have contributed to this improved performance.

The involvement of InsP₃ in other stress tolerance processes, beyond drought, has also been demonstrated. Alimohammadi et al. [15] obtained transgenic tomato plants (*Lycopersicon esculentum* cv. MicroTom,) overexpressing a human type I InsP₅-ptase gene. These plants presented a decreased level of InsP₃ and supported a continuous exposure to light longer than wild plants. Prolonged exposure to light causes oxidative stress in plant cells and can result in irreversible damage. However, the molecular mechanism involved in this tolerance process was not reported, but these transgenic lines were characterized in more detail by Alimoham-

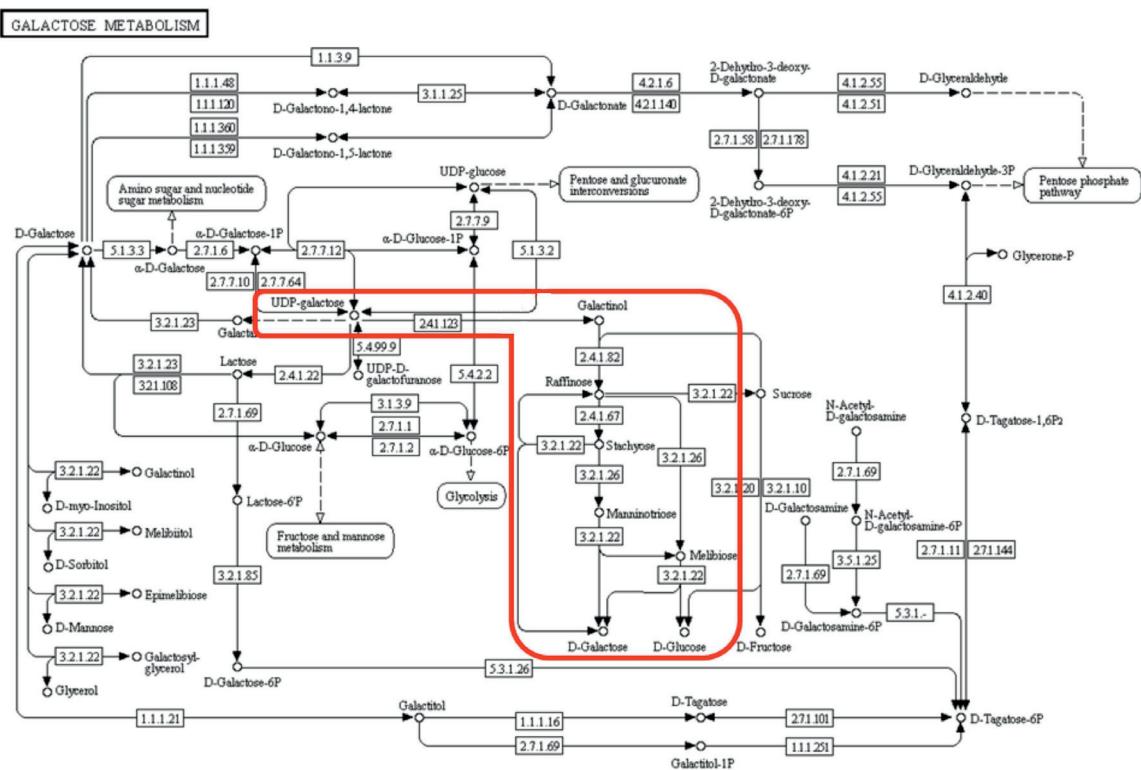


Figure 2. The Galactose metabolism from the KEGG Pathway database. Highlighted in red are the enzymatic reactions associated with the metabolism of Raffinose Family Oligosaccharides.

madi et al. [16]. These authors observed that under stress conditions, the transgenic plants maintained high chlorophyll content and accumulated low levels of hydrogen peroxide. This fact was attributed to the induction of genes related to multiple antioxidants [LeAPX1 (*L. esculentum* ascorbate peroxidase 1), SICAT2, LeSOD (*L. esculentum* superoxide dismutase)] during continuous exposure to light. Other effects included overexpression of the LePHYB photoreceptor (*L. esculentum* phytochrome B) and a key enzyme [LeCHS1 (*L. esculentum* chalcone synthase)] in the biosynthesis pathway of flavonoids, which are plant nonenzymatic antioxidants. There was also an overexpression of the SIMYB12 transcription factor, leading to an increase in flavonoids in tomato plants by up-regulation of the LeCHS1 expression. A relationship was established between change in phosphoinositol signaling pathway and increases tolerance to continuous exposure to light, through the activation of ROS-scavenging enzymes, and up-regulation of molecular activators of non-enzymatic antioxidants.

The biotechnological potential through the manipulation of compounds shown in Ins-related metabolic pathways may also be seen in the work of Ahmad et al. [17]. These authors performed a comprehensive analysis of *A. thaliana* genome, using the activation tagging technique in dedifferentiated calli, to identify salt-tolerant mutants (NaCl 150 mM). To this end, plants were modified with pRi35ADEn4 binary vector. Such vector contains four copies of the 339 bp long cauliflower mosaic virus (CaMV) 35S enhancer in the construct that induces the express-

sion of adjacent genes after proper insertion. Of the 18 potential tolerant mutants (150 mM NaCl), a line (stc1; salt-tolerant callus 1) presented the gene for myo-inositol-1-P-synthase-1 [At4g39800; MIPS1; EC 5.5.1.4; Figure 1] with induced expression in callus, with or without salt. This gene expressed 45 and 15 times higher compared to wild-type under the control condition. MIPS catalyzes the first step in the biosynthesis of inositol from glucose-6-P (Figure 1). The referred induction was greater in the mutant line than in the wild type (approximately, 260 times higher), both under stress. The *mips* gene transcription in the wild type reduced dramatically under stress condition. The tolerance analysis revealed that the mutant plants regenerated from calli showed salt tolerance in germination and growth. However, the mechanism involved was not disclosed, but the authors suggested that MIPS protect the calli and the plants from salt stress as osmolytes or by providing a precursor in the regulation of signal transduction pathways.

Kusuda et al. [18] went beyond the study of transformed lines overexpressing enzymes from the Ins-related pathways. They analyzed the differences among wild type and transformants lines in regard to salt tolerance in 3.5 days in medium with concentrations up to 250 mM NaCl. They also sought for differences by mining the metabolomes (the fourth leaf tissue harvested at 0, 6, and 12 h after NaCl stress induction) of the studied plants. To this end, a rice cultivar (*Oryza sativa* cv. Kitaake) was transformed with the construction Act::RINO1. The RINO1 gene encodes an MIPS (EC 5.5.1.4, Figure 1). It has been demonstrated that the constitutive overexpression of rice MIPS when compared to a wild type, results in greater tolerance to salt stress. Furthermore, it leads to a range of metabolic changes, with increased production of various metabolites (such as inositol, raffinose, ascorbate, amino acids). These handle the protection of plants from abiotic stresses. Additionally, activation of basal metabolisms such as glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle has been observed during induction of the Ins metabolism in those plants overexpressing MIPS.

Ins metabolism and phosphorylated Ins-derivatives are also associated with response to biotic stresses in plants. This fact shows the plurality of actions of these compounds. Murphy et al. [19] report evidence in this direction. They obtained transgenic potato [*S. tuberosum* L. (cv. Desiree)] and *A. thaliana* lines, synthesizing low levels of phytic acid (1-D-myo-inositol-P6 or InsP6, Figure 1). The transgenic potato lines were obtained by: (1) constitutive expression of an antisense sequence of the myo-inositol 3-phosphate synthase gene. This enzyme (IPS, EC 5.5.1.4, Figure 1) catalyzes the first step in the InsP6 biosynthesis; (2) plants expressing the *Escherichia coli* polyphosphate kinase (PPK, EC 2.7.4.1; Figure 1). PPK inserts inorganic phosphate into chains of phosphate residues linked by phospho-anhydride bonds, and this decreases the phosphate available to InsP6 biosynthesis. Genetically modified potatoes presenting both (1) and of (2) showed increased susceptibility to avirulent pathogen potato virus Y and the virulent pathogen tobacco mosaic virus (TMV). In relation to *A. thaliana*, the authors obtained three loss-of-function mutants [two (atips1 and atips2) involving the gene for IPS (EC 5.5.4.1, Figure 1); and one (atipk1) involving the gene encoding inositol polyphosphate kinase (IPK1; EC 2.7.1.158, Figure 1)]. IPK1 catalyzes the final step in InsP6 and without this enzyme, very little InsP6 is done. The disruption of InsP6 biosynthesis in *A. thaliana* resulted in an increased susceptibility to viruses (tobacco mosaic virus), bacteria [*Pseudomonas syringae* pv tomato (Pst) DC3000 and Pst DC3000 AvrB], and fungus (*Botrytis cinerea*). The

increased susceptibility only occurred for *atips2* and *atipk1* mutants. For *atips1* mutants showing also InsP6 depletion, the resistance to pathogens was not compromised. This suggests either that a particular pool of InsP6 regulates defense against pathogens in *A. thaliana*, or IPS1 and IPS2 are differentially regulated, and one cannot compensate for the other. It was observed with regard to the typical responses of both species that levels of salicylic acid (SA), a key molecular signal for establishing local and systemic acquired resistance, were not diminished. Therefore, SA was not responsible for increased susceptibility to pathogens analyzed. Furthermore, a relationship between the inositol phosphate metabolism and basal resistance to pathogens (fungi, bacteria, and viruses) has been established. This indicates that InsP6 is also required as an essential operation signal for regulation and defense in plants, together with several other well-known defensive signals (NO, cADPR, SA and Ca²⁺).

Recently, Meng et al. [20] found that *A. thaliana* *ips1* loss-of-function mutant (*atips1*; IPS; EC 5.5.4.1, Figure 1) exhibits spontaneous cellular death and increased resistance to oomycete *Hyaloperonospora arabidopsis* pathogen. This result, together with the above, supports the premise that a particular InsP6 pool may regulate defense pathways, since *atips1* mutants did not show increased susceptibility to the variety of testes pathogens (Tobacco mosaic virus; *Pseudomonas syringae* pv. tomato (Pst) DC3000 and Pst. DC3000 AvrB; and *Botrytis cinerea*). Additionally, it was observed that spraying the *atips1* mutant plants with Ins could suppress the formation of spontaneous lesions, indicating that Ins production and not AtIPS1 protein is required to prevent the lesion formation. The authors also found evidence for the role of Ins (or Ins-derivatives) in the regulation of programmed cell death.

Reports presented in Table 1 also show the broad roles of Ins and its phosphorylated derivatives in plant cells. From the wide spectrum of analyzed genes, a range of effects on plants at different levels was observed. These effects have shown associations with hormone signaling pathways, such as ABA [12], influences in photosystems [21, 22], with reactive oxygen species (ROS; [23]), with relative water content, with osmotic adjustment [24], among others (Table 1).

3. Methylated Ins-derivatives in plant cell and the biotechnological use to increase stress tolerance

Some plants use Ins as precursor of compatible solutes such as D-ononitol and D-pinitol, which act as osmoprotectants (small molecules that act as osmolytes and help organisms survive in extreme osmotic stress [35]). In halophyte ice plant (*Mesembryanthemum crystallinum*), which is considered highly tolerant to drought, salinity, and cold, Ins is methylated to D-ononitol and subsequently epimerized to D-pinitol [36]. The myo-inositol O-methyl transferase gene (IMT1; EC 2.1.1.40; Figure 1) is transcriptionally induced by osmotic stress, whereas neither transcriptional nor enzyme activities is detectable in ice plants under normal growth conditions [37, 38]. Despite the positive influence of these metabolites in plant physiology under abiotic stress conditions, there are less available data for these compounds compared with phosphorylated Ins-derivatives.

EC† Number	Gene origin (specie)	Transformant (specie)	Gene Modulation	Analysed Condition	Impact on Tolerance	Authors	Notes
5.5.1.4	<i>Pc</i>	<i>Os and Bj</i>	Overexp.	HS	Raise	[25]	a.
5.5.1.4	<i>Pc</i>	<i>Nt</i>	Overexp.	HS	Raise	[21]	b.
5.5.1.4	<i>As</i>	<i>At</i>	Overexp.	HS	Raise	[22]	c.
3.1.3.25	<i>Ca</i>	<i>At</i>	Overexp.	HS, P, PEG, and HT	Raise	[26]	d.
2.7.1.140; 2.7.1.151	<i>At</i>	<i>Nt</i>	Overexp.	HS and OS	Raise	[27]	e.
2.7.1.140; 2.7.1.151	<i>Th</i>	<i>Bn</i>	Overexp.	HS, D, and OS	Raise	[28]	f.
3.1.4.11	<i>Zm</i>	<i>Zm</i>	Overexp.	D	Raise	[29]	g.
2.7.1.159; 2.7.1.134	<i>Os</i>	<i>Nt</i>	Overexp.	HS	Decrease	[24]	h.
3.1.3.57	<i>At</i>	<i>At</i>	Knockout	HS, F, and D	Decrease	[30]	i.
2.7.1.137	<i>At</i>	<i>At</i>	Knockout	HS	Decrease	[31]	j.
2.7.1.67	<i>At</i>	<i>At</i>	Overexp.	HS and ABA	Raise	[23]	l.
2.7.8.11	<i>Zm</i>	<i>Zm</i>	Overexp.	D	Raise	[32]	m.
3.1.3.8	<i>At</i>	<i>At</i>	Overexp.	HS and OSM	Raise	[33]	n.
2.7.1.149	<i>At</i>	<i>At</i>	Overexp.	HS, D, and ABA	**	[34]	-

†Enzyme Commission; *According to KEGG Database Pathway; – Not observed; Overexp. (Overexpression); **Legend:** HS (high salinity); P (paraquat); PEG (polyethylene glycol); HT (high temperature); OS (oxidative stress); D (drought); DH (dehydration); ABA (ABA hormone); OSM (osmotic stress); F (freezing). **a.** Albeit to a variable extent, overexpression of this gene confers salt-tolerance to diverse evolutionary organisms (from prokaryotes to eukaryotes), including crop plants; **b.** Transgenic individuals presenting retention of approximately 40–80% of the photosynthetic competence under analyzed stress condition; **c.** Transgenic individuals retained more chlorophyll and carotenoid by protecting the photosystem II; **d.** Improving seed germination and seedling growth in transgenic individuals under stress conditions; **e.** Expression patterns of various stress responsive genes were enhanced, and the activities of antioxidative enzymes were elevated in transgenic plants; **f.** The transcripts of various stress-responsive genes are increased in ThIPK2 transgenic plants under salt stress condition; **g.** The sense transgenic plants had higher relative water content, better osmotic adjustment, increased photosynthesis rates, lower percentage of ion leakage and less lipid membrane peroxidation, higher grain yield than the wild type; **h.** The 1,3,4-trisphosphate 5/6-kinase is a negative regulator of osmotic stress signaling in tobacco; **i.** The genetic evidence indicating that phosphoinositols mediate ABA and stress signal transduction in plants, and their turnover is critical for attenuating ABA and stress signaling; **j.** Salt stress responses, such as increased plasma membrane endocytosis and the intracellular production of ROS, are coordinated by phospholipid-regulated signaling pathways; **l.** AtPI4Kγ3 is activated by DNA demethylation and regulates the ROS accumulation induced by high salt treatment or ABA treatment; **m.** ZmPIS regulates the plant response to drought stress through altering membrane lipid composition and increasing ABA synthesis in maize; **n.** AtPAP15 (3-PHYTASE) may modulate AsA levels by controlling the input of myoinositol into this branch of AsA biosynthesis in *Arabidopsis thaliana*. *At*: *A. thaliana*; *Pc*: *P. coarctata*; *Sa*: *S. alterniflora*; *Ca*: *C. arietinum*; *Th*: *T. halophile*; *Zm*: *Z. mays*; *Os*: *O. sativa*; *Nt*: *N. tabacum*; *Bn*: *B. napus*; *Bj*: *B. juncea*.

Table 1. Transgenic and knockout plant assays available in the literature related to (poly)phosphoinositides and inositol (poly)phosphates. Relevant information: EC number of the enzyme coded by the studied gene, plant donor species, the genetically modified organisms (transformants), the modulation of the studied gene, the analyzed stress condition, the impact on plant tolerance and physiology (additional details, please see the legends).

Sheveleva et al. [39] were one of the first to report the biotechnological potential of methylated Ins-derivatives. In their work, the authors supereexpressed O-methyltransferase (IMT1; EC

2.1.1.40; Figure 1) of *Mesembryanthemum crystallinum* in tobacco (*Nicotiana tabacum* cv. SRI). The transgenic plant increased its tolerance to abiotic stresses [drought and high salinity (50–250 mM NaCl)] when compared to the wild control line. An accumulation of methylated inositol D-ononitol in amounts of fresh weight exceeding 35 $\mu\text{mol g}^{-1}$ in the transformed lines was observed. Besides, the photosynthetic CO₂ fixation was less inhibited in those plants during drought and salt stress. Further, transformed plants recovered faster than the wild type after rehydration. In turn, Sengupta et al. [40] observed an increase of the D-pinitol synthesis in a wild-type rice (*Porteresia coarctata*) with halophilic characteristics when subjected to high salt environment (400 mM NaCl). An increment also occurred in both transcriptional and proteomic level of IMT1, not observable in domesticated rice under the same condition. The authors also reported an increase in the expression of L-myo-inositol 1-phosphate synthase (PcMIPS1; EC 5.5.1.4; Figure 1), along with the expression of IMT1. According to the authors, this suggests that the accumulation of D-pinitol would be a mechanism regulated by salt stress.

Recently, Zhu et al. [41] used a similar strategy as the one developed by Sheveleva et al. [39] to express in *A. thaliana*, a gene coding IMT1, from *Mesembryanthemum crystallinum*. The transformed plants showed higher growth compared to the wild control line and increased tolerance to cold stress (4°C). This increment in tolerance was attributed to different factors: (1) the electrolyte leakage content in the transgenic plants was significantly lower than that of the wild-type plants after freezing stress, showing less damage to the membranes of those plants; (2) transgenic plants showed lower MDA content than wild-type plants, not only in normal conditions but also after stress; and (3) a higher proline content presented in transgenic lines than in wild type, after application of stress.

4. Oxygenated Ins-derivatives in plant cell and the plant strategy to tackle stress

So far, the myo-inositol oxygenase (MIOX; EC 1.13.99.1; Figure 1) is the only enzyme known by the oxidation of Ins [42]. Its importance in plants stood out from the statement in the Ins metabolism as a precursor in Ascorbic Acid (AsA) biosynthesis in *Arabidopsis*. In this sense, Lorence et al. [43] observed the expression of a myo-inositol oxygenase (miox4) increasing the content of AsA in leaves (approximately two- to threefold). By that, they anticipated a potential use of the gene by genetic engineering, enhancing levels of this important antioxidant in plants. Further analysis indicated that D-GlcUA (Figure 1), a derivative from MIOX reaction (EC 1.13.99.1, Figure 1), plays a negligible role for AsA biosynthesis [44]. However, MIOX can control the metabolite level of myo-inositol in plants [44].

Nevertheless, the metabolic consequences of MIOX action are still unclear. In order to contribute with information from gene regulation and catalytic activity of this enzyme, Duan et al. [6] performed a functional characterization in rice (*Oryza sativa*), observing its predominant expression in root, with induced transcription under drought stress (20% PEG6000 solution), H₂O₂, high salt (200 mM NaCl), cold (4°C) and Abscisic Acid (100 μM). Transgenic rice lines overexpressing an MIOX gene showed a higher survival rate than a wild line, when in contact with 20% PEG6000 solution. In the same way, the authors also reported for the

transgenic lines, transcription levels significantly induced for genes coding enzymes associated with ROS scavenging, suggesting an MIOX activity in reducing oxidative stress.

Moreover, Alford et al. [45] reported MIOX enzymes responding to growing conditions of *A. thaliana* in low energy/nutrient environment. Their data supported the hypothesis that MIOX2 and MIOX4 enzymes would be encoded by multiple genes. Furthermore, on plants growing in low energy conditions/nutrients environments, MIOX2 enzyme would have a significant role in providing Ins to many different tissues, whereas MIOX4 would act in supplemental form at some tissues. An analysis of promoters was presented, considering multiple lines of MIOX2p:GUS and MIOX4p:GUS seedlings growing on: (I) no nutrients (agar); (II) low nutrients (agar + 0.5× MS salts); and (III) optimal nutrients (agar + 0.5× MS salts + 3% glucose) in low light (40 μE) during seven days. As a result, only MIOX2p:GUS was expressed abundantly in condition I, whereas in condition II, both MIOX2p:GUS and MIOX4p:GUS showed moderate expression, having been more expressed previously.

5. Raffinose Family Oligosaccharides (RFOs) and plant strategy to address stresses

RFOs are a class of compatible solutes coming from Ins metabolism ramifications. As mentioned before, the enzyme GolS (EC 2.4.1.123, highlighted in red in Figure 2) connects the metabolism of these compounds, producing galactinol (highlighted in red in Figure 2), which serves as galactose donor for further synthesis of RFO members [8, 9]. To date, structural genomics data and global transcriptome analysis concerning RFOs are only available for corn [46]. For this crop, the authors have performed a genomic identification of genes associated with raffinose metabolism, together with an expression analysis using data-mining from GEO (<http://www.ncbi.nlm.nih.gov/geo>) and PLEXdb databases (<http://www.plexdb.org>). Additionally some transgenic lines overexpressing specific gene isoforms related to RFO pathway, under particular growth conditions, are available for some species. These studies showed a positive impact in some crops.

Taji et al. [47], for example, analyzed the expression of seven genes encoding GolS in *A. thaliana* under different stresses. From those genes, only three (AtGolS1, 2 and 3) were stress-responsive. AtGolS1 and AtGolS2 were induced by drought and high salt (250 mM NaCl), but not by low temperature (4°C). On the other hand, AtGolS3 was induced by low temperatures, but not high salinity or drought. The AtGolS2 overexpression in *A. thaliana* was associated with an increase of raffinose and galactinol and resulted in a reduction of leaf transpiration with a greater drought tolerance. In turn, Dos Santos et al. [48] analyzing three *Coffea arabica* galactinol synthase isoforms (CaGolS1, CaGolS2, CaGolS3) observed a mainly tissue-specific expression but differentiated regulation depending on the applied stress (drought, heat, and high salinity). This reinforces the observation by Taji et al. [47], indicating that different galactinol sets can be necessary for response to various stresses. The GOLS (BnGOLS-1) activity was also positively correlated with desiccation tolerance in cabbage seeds (*Brassica napus*) during the vegetative growth period [8]. The tolerance was observed around 21-24 days after flowering cabbage, coinciding with the accumulation of raffinose and stachyose. The

BnGOLS-1 transcripts accumulation was concomitant with the formation of these two RFO members [8].

In *Arabidopsis thaliana* leaves overexpressing HsfA2 (Heat-Shock Transcription Factor A2; [49]) was also found highly induced GolS1, -2, -4 and Raffinose Syntase 2 (RS2; EC 2.4.1.82, highlighted in red in Figure 2) transcriptions. The galactinol and raffinose levels in the transgenic plants were higher compared to the wild-type lines, both in the control condition. These higher levels were positively correlated with an increase in plant tolerance when exposed to the studied stresses [mevalonate (50 mM), high salt (100 mM NaCl) and low temperatures (4°C)].

Latter, Pennycooke et al. [50] studied the expression of α -galactosidase gene (EC 3.1.2.22 highlighted in red in Figure 2) from petunia (*Petunia x hibrida* "Mitchell"), monitoring acclimated plants to low temperatures (4°C) and in response to increasing temperature (25°C). Transcripts induction were observed after one hour of desacclimation occurring together with an increase in enzymatic activity and decreased raffinose content, suggesting that the rise in temperature can regulate the RFO catabolism of certain members, through gene regulation that encoding α -galactosidase.

Thus, the diversity of functions performed by compounds presenting in the Ins metabolism was shown in the described works. Also, studies of distinct isoforms showed positive correlations with plant responses to various abiotic stresses. In this way, the identification of new transcripts, as well as the understanding of its regulation (spatial and temporal) in plants under unfavorable conditions for the development may lead to the discovery of new genes with biotechnological potential.

According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (<http://www.genome.jp/kegg/pathway.html>), which provides diagrams of various metabolic processes, at least 45 enzymes are associated with the metabolic pathways described here. Of these enzymes, 21 (highlighted in green boxes in Figures 3A and 3B) have been studied in previous works addressing the transcriptional expression of their genes or effects on plants under stress. Therefore, there are at least 24 enzymes (in red boxes in Figures 3A and 3B) that have not been targets of these analyses, with significant potential for further research in biotechnology.

6. Ins and its derivatives in humans: Antinutrients *versus* disease prevention

Once Ins and its derivatives are present in vegetables and these are part of the daily diet of large populations around the world, it is essential to analyze their potential effects on consumers. The Ins and related metabolites play a heterogeneous physiological role, depending on the concerned organism, plant or animal (including human). In plants, as already mentioned, such compounds help regulate plant homeostasis during periods of stress. In animals (including humans), their influence has very diverse physiological repercussions. Initially, they were only seen as harmful agents because some representatives when present in certain

plants could act as antinutritional factors, thereby reducing the bioavailability of important nutrients and the nutritional value of the food. According to Kokhar and Aperten [51], this effect is present a result of a selected adaptive mechanism due to a "chemical warfare" between higher plants and herbivorous pests.

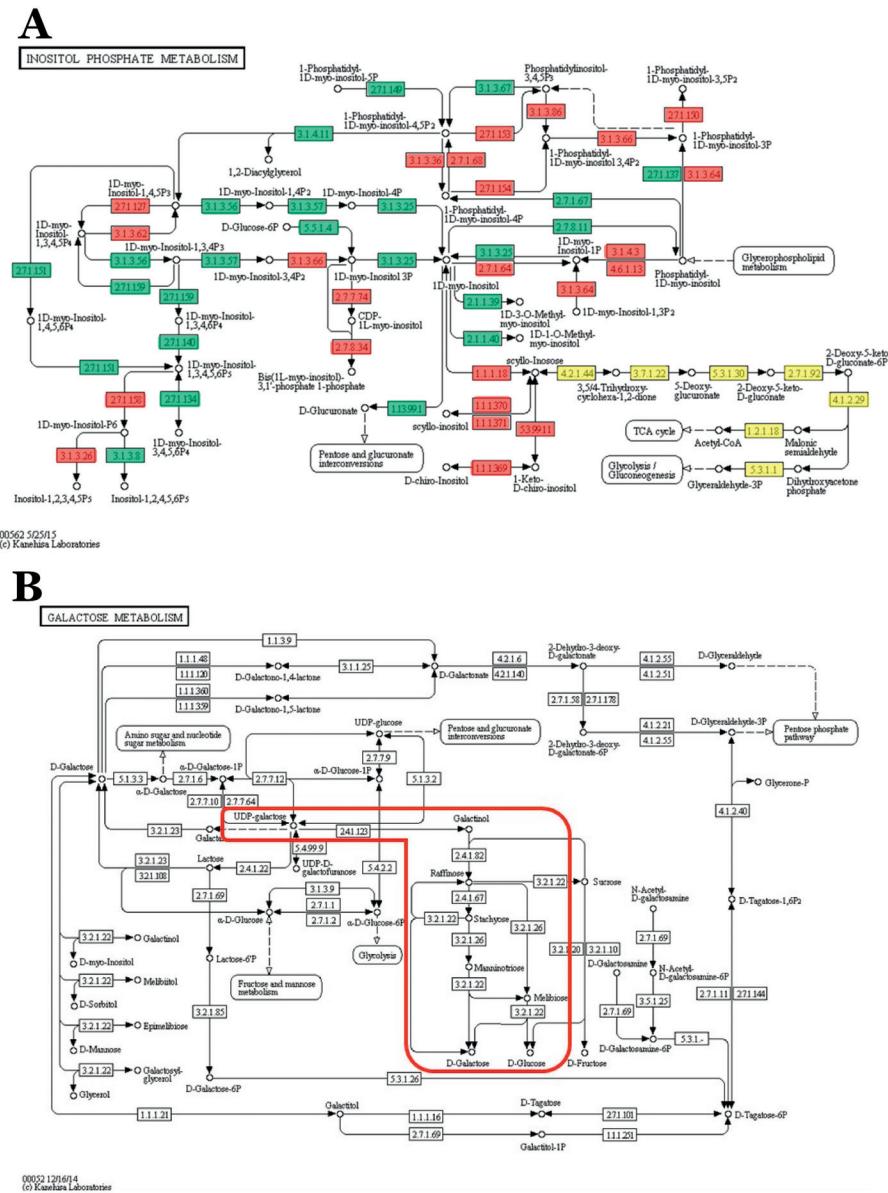


Figure 3. KEGG Pathway database diagrams presenting: (A) Inositol Metabolism; (B) Galactose Metabolism, showing the Raffinose Family Oligosaccharides (highlighted in red). Green Box: enzymes with transcriptional modulation data available from stress assays. Red Box: enzymes without transcriptional modulation data available in the literature. Yellow Box: enzymes not covered in this review.

Among the various Ins-derivatives, phytic acid (1-D-myo-inositol-P₆; Figure 1) is the most studied, concerning the impacts on human and animal health. Its unique structure provides the ability to chelate cations such as iron, zinc, potassium, magnesium, and copper, forming

insoluble salts denominated phytate. These salts adversely affect animal's ability to absorb and digest nutrients [52, 53]. Phytates can form complexes with proteins, changing their structures and their enzymatic activities and characteristics of solubility and proteolytic digestibility [54]. However, there are reports that show positive aspects in phytates consumption. The presence of these in the diet of patients with diabetes has positive effects in reducing the level of blood glucose due to decreased starch digestion rate and slowing of gastric evacuation [55]. There are also reports of activity against HIV replication, kidney stones prevention, reduction of cholesterol and triglycerides levels, as well as assistance in prevention of heart diseases (for review see [54]). Studies also indicate that both Ins [56] and phytic acid [56, 57] have anticancer properties. With regard to RFOs, besides the fact that they are potential antinutritional factors, there are indications that they may act as important immunostimulants in animals (including humans). Also, RFOs' involvement is suggested in universal mechanisms of oxidative balance in several taxa [58].

7. Concluding remarks and perspectives

Experimentally, mutants and transgenic analyses are being successfully carried out to uncover the various roles played by Ins-related compounds. It is known today that some phosphorylated derivatives of inositol are connected with a large number of signaling procedures which are regulated by both abiotic and biotic stress. Methylated and oxygenated Ins-derivatives, including RFOs, have also proven to be active agents in the process of plant acclimatization to unfavorable conditions, involved in a number of functions. However, there is a gap to be filled. About two dozen enzymes associated with the synthesis of these compounds have not been adequately studied and they represent an area of high biotechnological potential.

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Plant Integrity – The Important Factor of Adaptability to Stress Conditions

Ladislav Bláha and Tomáš Středa

Abstract

Crop production, research of crop productivity, tolerance to abiotic and biotic stresses, plant disease, and pests all represent the problem of plant integrity. Plants represent an integrated system of units, which are responsible for its resistance to adverse environmental conditions on the basis of the evaluation of characteristics both aboveground and in the roots. This "complete unit" (root and shoot) has an influence on the formation of seeds, the quality of which may affect subsequent growth, development, and stress tolerance of the filial generation. Properties of the roots predominantly influence (especially at drought stress conditions) growth, development, and the metabolic processes in the aboveground part of the plant. The seed traits affect the filial generation root morphology at the beginning of the vegetation period (especially length, surface, depth of root penetration, and also root weight). In the biology of the seeds, roots, yield formation, stress tolerance, etc., attention needs to be paid to plant integrity and adaptability during variable environmental conditions. Every plant, and its traits, is a result of all the plant's activities. This is important for plant breeding. For example, it is possible to provide selection for cultivar traits at seed germination. Quality of the embryonic traits is important for subsequent growth and development. In the juvenile phase, and in later stages, the same genotype is still active. This is among the main reasons for studying plant integrity.

Keywords: Plant integrity, abiotic stress, crop production, weather variability, seeds, roots

1. Introduction

The effect of plant integrity and of aboveground/belowground defense signaling on plant resistance to abiotic and biotic stresses is a basic and very important subject of contemporary scientific research.

Profitable, stable crop varieties demonstrate superior traits for all types of observed characteristics in all environmental conditions (seeds, roots, shoots). Good varieties in ecologically different conditions usually have superior features for all types of analyzed traits. It is possible to state on the basis of whole plant metabolism, that plants create integrated system units, which are responsible for resistance to adverse environmental conditions on the basis of evaluation of characteristics of their aboveground parts and their roots. This "unit" (root and shoot) has an influence on the formation of seeds, which, by their quality, may affect the subsequent growth and development of the filial generation, and thus is also resistant to external conditions.

The earliest information about "physiological manifestations of plant integrity" was found in ancient Rome, in the era of Gaius Julius Caesar. Plant integrity, regarding the "functionally coordinated whole plant body", was first defined on the basis of scientific knowledge in the Czech Republic by Rudolf Dostál (1885–1973). Properties of the roots significantly affect (especially during drought conditions) growth, development in the aboveground part of the plant, and plant seed growth and development. From the opposite viewpoint, it is known that seed traits affect, in the filial generation, root morphology at the beginning of the vegetation period: especially length, surface, depth of root penetration, and also root weight.

The first description of seeds was presented by the Greek scholar Theophrastus (372–282 B.C.). The main development of seed science began in the 19th century [1]. Seed traits are factors, with different levels of importance, which are given by soil and weather conditions during the sowing and time of emergence. The processes and traits of germination and field emergence of a plant are very important for subsequent growth and development. Genotypes with good seed germination under unfavorable conditions develop, in the filial generation, larger root systems in field conditions. However, the most physiological experiments with green parts of plants do not take into account the fact that "half" of the metabolic processes in plants take place in the roots. This part of the plant has an important influence on the shoot traits and seed production.

Darwin expressed that "roots are as brain of plants", i.e. roots can be taken as a similar body to the brain. Currently, it is known that for transmission signals (changes of potential) between roots and the aboveground parts of the plant, plasmodesma are needed. The root system provides transmission information to other parts of the plant (shoots and seeds under development). Information about pathogen attack or physical stress can be transmitted to the other parts of the plant in order to begin as soon as possible the organisms defense – thanks again to plasmodesma. If the stress is repeated again, the reaction of the plant is more rapid, based upon "plant memory".

In the biology of the seed, especially in seed productions we need to pay attention to plant integrity and adaptability over variable environmental conditions. Adaptability (plasticity) is a feature that allows the plant to return to its original previous physiological state, i.e. to basic metabolic functions, after unusual environmental conditions. Adaptability is fixed on all chromosomes and can be identified in the early stages of growth. Adaptability in a broader sense includes seed quality, especially the quality of sprouting plants, root system adaptability, and issues relating postharvest physiological processes, including the physiological processes

during storage. On the basis of entire plant physiology, it is possible to use more than 100 plant characteristics to improve adaptability under variable environmental conditions.

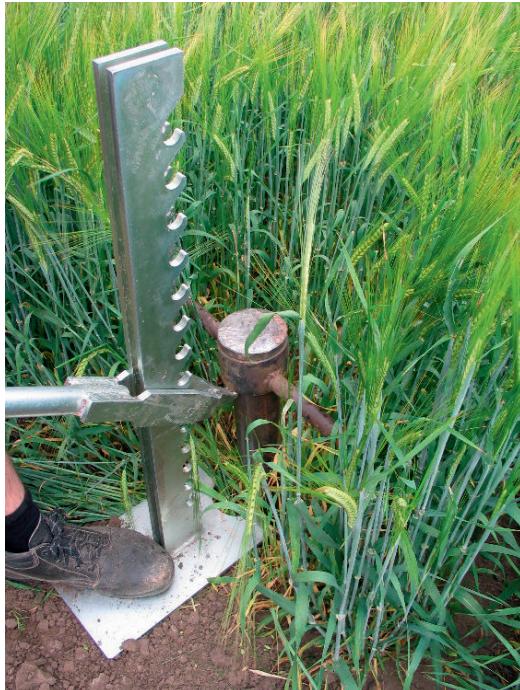


Figure 1. Sampling the root biomass for the evaluation of the relation between above and below ground parts of a plant.



Figure 2. Plant roots in the interior of Kateřinská Cave (Moravian Karst, Czech Republic).

2. Main abiotic plant stresses in central Europe

Abiotic stress is a main cause of reduced yield in the case of healthy plants. In this context, many scientific research projects have dealt with the impact of weather and climate change on agricultural crops. They were primarily focused on the impact of stress evaluation – especially drought and air and soil temperature extremes. The main current problems are lack of soil water or soil drought as well as high air temperatures. It has also been proved that there has been a prolongation of the growing season – 15 to 25 days – in central Europe in the last 20 years. This is accompanied also by an increasing probability of risk of vegetation frost. Concrete adaptation measures that would eliminate the impacts of climate change are still not a reality.

Monitoring of meteorological elements is crucial for the precise description of microclimatic conditions and their influence on plant physiological processes. The outcomes of microclimate monitoring provide valuable data for growth, plant protection, yield, and irrigation models as well as a wide range of other applications. Monitoring of air temperature and humidity as well as temperature and soil moisture and solar radiation should be an integral part of all growing trials – pot or field based. It is difficult to find any kind of cultivation experiment where the soil moisture and air temperature do not play important roles.

Globally, agriculture accounts for 80–90% of the freshwater used by humans. In many crop production systems such a water use is unsustainable. An interdisciplinary approach involving agronomical opportunities and plant breeding in order to deliver “more crop per drop” is needed [2]. In the field, the upper limit of water productivity for well-managed, disease-free, water-limited cereal crops is typically $20 \text{ kg ha}^{-1} \text{ mm}^{-1}$ (grain yield per water used). Climate development in Europe since 1990 has been unfavorable for cereal yields because of heat stress during grain filling and drought during stem elongation. Drought during the generative phase decreases the number of based spikelets and grains. Another critical period is also flowering, when water shortage impact is worse than during other stages of development. During the stage of seed filling water stress disrupts the process of synthesis and storage of starch and storage proteins. It has been confirmed [3] that early drought reduces the number of offshoots and number of grains per ear. Late drought at the time of the development of leaves and grain filling causes leaves to age and their photosynthetically active surface decreases faster than in irrigated plants. Late drought negatively affects grain size.

Spring cereal yields decreased by 45–75 kg ha^{-1} due to decreased precipitation of 10 mm [4]. The highest values of water requirement in plants were observed in the stages from shooting to heading, during an intensive increase of biomass. During this period, the plants utilized up to 5 mm of water per day. Seasonal deficits of precipitation during the growing season in central Poland were -145 and -169 mm for barley and wheat, respectively. In the growing season they utilized from 293 to 314 mm of the soil water [5].

Moisture certainty analyses in the Czech Republic (central Europe) proved there was an increase in the driest areas and that drought event probability increased in during the 1961–2010 period [6]. An increase in air temperature above normal months and the loss of normal precipitation months were identified. An increase in temperature and precipitation extremes

in the future, across climatic conditions and types of landscapes in the Czech Republic, was found [7]. The occurrence of meteorological drought, as well as the occurrence of hydrological, agronomic, physiological, socio-economic, and other kinds of drought is an important feature of the Czech climate. Lack of soil moisture is expected in the main growing season (approximately 200 days) when the rainfall does not exceed 340 mm. In connection with the stress effects on yield and quality the most important indication for growers is the presence of agronomic drought. This is defined as a state where the amount of moisture in the soil is less than that required by a particular plant. Literature often defines agronomic drought as a decline in soil moisture below the permanent wilting point (i.e., approximately -1.5 MPa) which stops water uptake and, subsequently, plants growing. It has been claimed [8] that the proportion of usable water – not reducing yields – varies according to crop type and stage of development between 45% and 75% of the available water holding capacity (AWHC). [9] use soil moisture in the root zone at 65% AWHC as a limiting value for barley before transpiration is reduced.

The availability of soil water, together with global radiation belong to the main agrometeorological elements which determine the transpiration performance of plants. Global radiation has a primary effect on the transpiration of plants, however, in the case of drought stress occurrence, one may expect a major influence to be played by soil moisture on the course of transpiration. A crop's reaction to a decrease in soil water capacity is different for different crop species. The high evapotranspiration requirements of the environment may cause a loss of soil water through excessive transpiration in non-sensitive plants.

Water shortage-induced stress often goes hand in hand with temperature stress. Transpiration is the main mechanism a plant has to protect itself against overheating. Leaf temperature increases with increase in air temperature. Effective use of water implies maximal soil moisture capture for transpiration, which also involves reduced non-stomatal transpiration and minimal water loss by soil evapotranspiration.

The dependence of maize transpiration on air temperature, air humidity, solar radiation, soil moisture, wind speed, and leaf temperature were quantified [10]. Significant relationships between transpiration, global radiation, and air temperature were found. Conclusive dependence of transpiration on leaf surface temperature and wind speed was found (Fig. 3–6). Transpiration in maize plants was significantly influenced by soil moisture under moderate and severe drought stress. The dependence of transpiration on meteorological elements decreased with increasing deficiency of water. A correlation between transpiration and plant dry matter weight, plant height, and weight of corn cob was found. These results will be utilized in an effort to make the calculations of evapotranspiration in computing models more accurate.

Breeding for maximal soil moisture capture for transpiration is therefore the most important target for yield improvement under drought stress. Conclusions have been made [11] that differences in the effective use of water expressed as different yields under the same conditions can be partly attributed to different root system sizes (RSS) (probably due to deeper rooting) and can be improved by breeding. A value of 55% AWHC [12] has been suggested as a

qualitative and not stressful value for all growth phases except at the beginning of flowering (45%) and plant maturation.

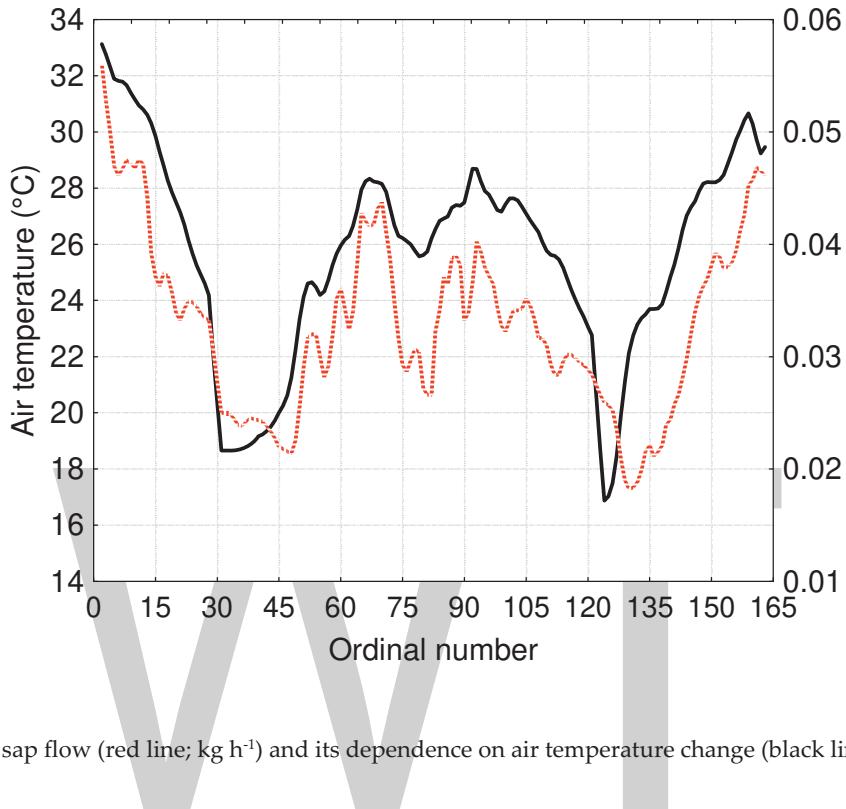


Figure 3. Course of sap flow (red line; kg h^{-1}) and its dependence on air temperature change (black line; $^{\circ}\text{C}$).

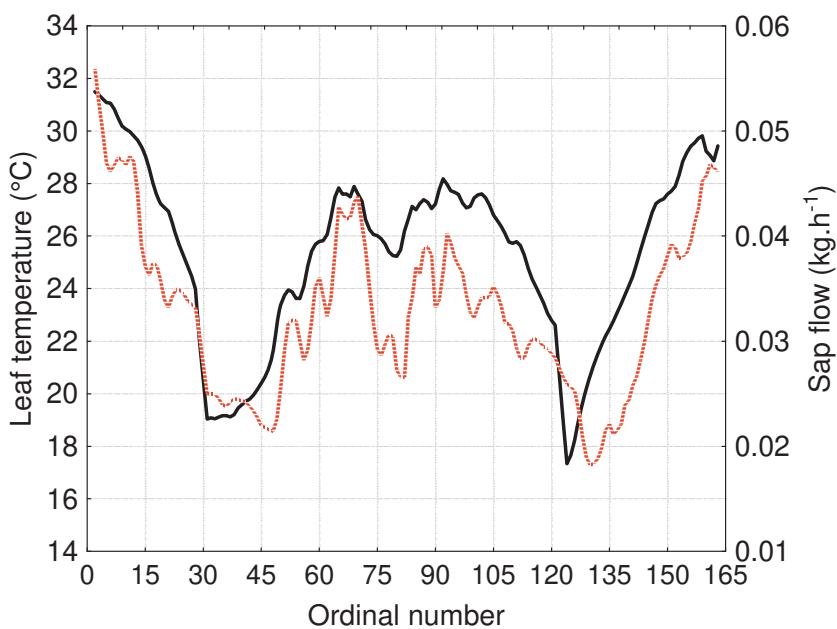


Figure 4. Course of sap flow (red line; kg h^{-1}) and its dependence on leaf surface temperature changes (black line; $^{\circ}\text{C}$).

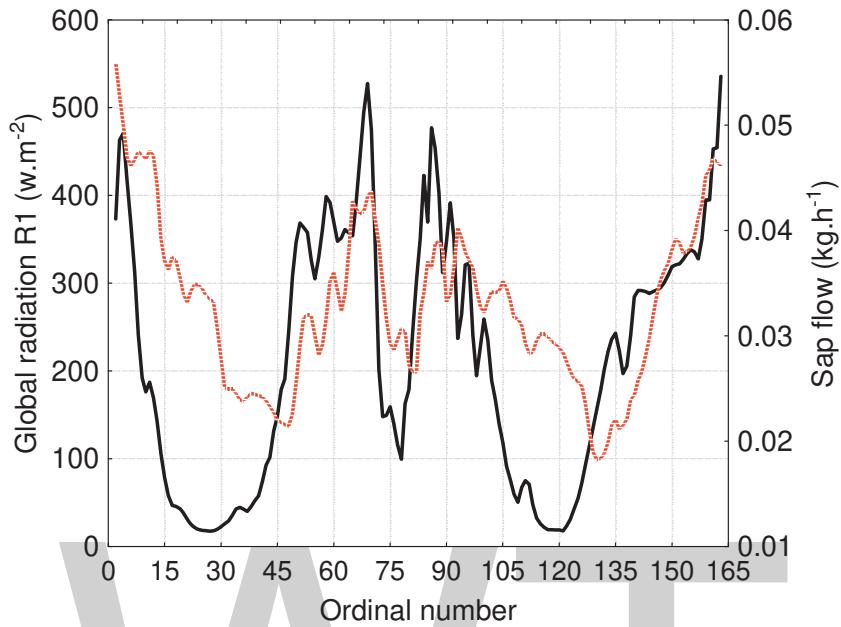


Figure 5. Course of sap flow (red line; kg h^{-1}) its dependence on global solar radiation intensity (black line; W m^{-2}).

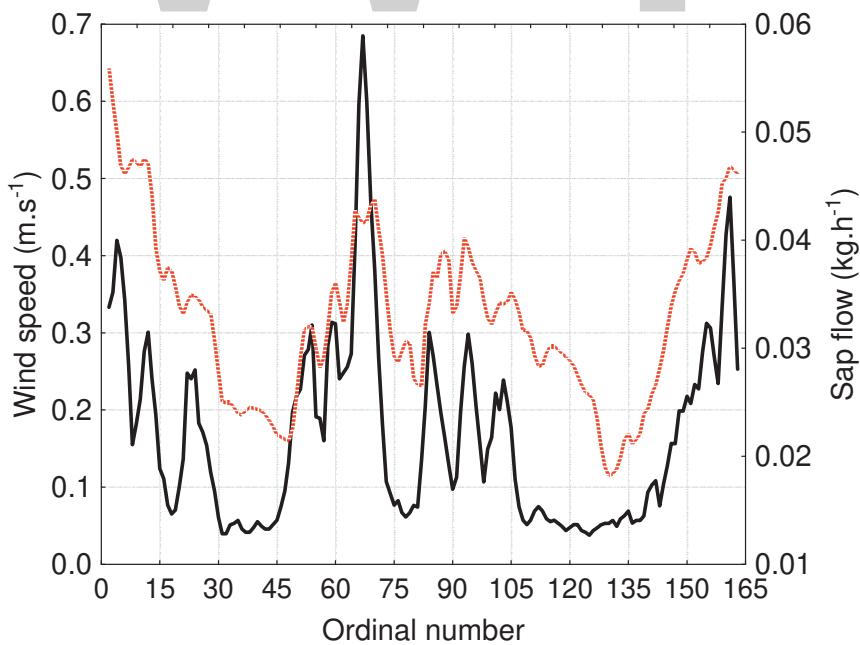


Figure 6. Course of sap flow (red line; kg h^{-1}) and its dependence on wind speed (black line; m s^{-1}).

The amount of usable soil water was calculated using the agrometeorological model AVISO at 21 experimental sites for the period 1975–2007 (% AWHC) [13]. A decrease in usable soil water (% AWHC decrease up to 24%) in a growing season was observed at 20 localities in the long-term trend. Statistically significant relationships were found between grain yield of spring barley and level of AWHC (% AWHC). The optimum range for the amount of usable soil water for the production of spring barley (65%–75% AWHC) was defined by long-term calculations of soil water in combination with a series of yield trials (Fig. 7).

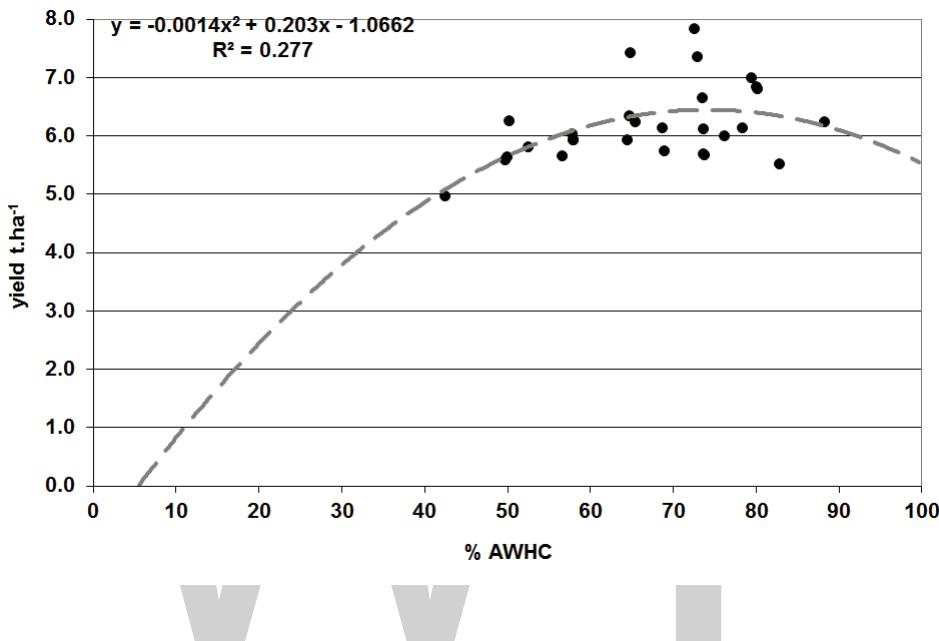


Figure 7. Relationship between the soil water supply (% AWHC) and yield of spring barley grain.

Decreasing winter precipitation, increasing winter air temperatures, and increasing levels of CO₂ in atmosphere were forecast as global climate changes for central Europe. The negative effects of water stress were partially compensated for by elevated CO₂ concentration. Warmer winters could lead to northward expansion of the areas suitable for cropping. However, for crops with a determinate growth habit (e.g. cereals) acceleration of development under warmer conditions could reduce the time available for growth before maturity thereby tending to reduce grain yield. Combining these effects with the fertilizing effect of increasing atmospheric CO₂ concentration, yield of wheat could be 30%–55% higher if there is enough water [14]. For non-determinate crops (e.g. root crops) the warmer climate would extend the growing season. However, there is the possibility that the more frequent, damagingly high summer temperature events could reduce yields of both cereal and root crops. Water can be limiting not only due to global warming but also due to higher yields caused by new varieties and by higher levels of agronomic inputs. Breeding for greater RSS could be therefore one of the strategies for avoiding the impact of water stress. For example, the grain yield of winter wheat varieties in dry years is generally positively correlated with RSS. In a dry year, the varieties that showed the greatest difference in RSS were found to exhibit a yield difference of 860 kg ha⁻¹, approximately translating to an additional use of 15 mm of subsoil water [11].

3. Reasons why it is necessary to consider the integrity of plants in agriculture research

3.1. Seed vigor

Seedling establishment is critical for production especially in stressed environments. The methods for the evaluation of seed germination are designed to have high levels of reproducibility and reliability; but worse than optimal conditions are often encountered in the field. For example, the germination percentage of barley (*Hordeum vulgare* L.) is an important character for both seed quality and malting quality. Although it is evaluated under optimal conditions for germination (laboratory temperatures and optimal moisture), it differs from germination in the soil and malting. In addition, seed lots that do not differ in germination may differ in emergence and storage potential. Seed vigor is generally described as the sum of the seed properties that determine the potential level of activity and performance of the seed during germination and seedling emergence [15]. Seed vigor is the ability of seeds to germinate and form the basis for future plant growth and development in standard and stressed conditions (drought, low temperatures, lack of nutrients). When soil conditions were unfavorable, the results of field emergence for wheat were more closely correlated with the direct stress vigor tests than laboratory germination [16]. The expression of seed vigor in field conditions and the translation to higher yields depends on the environment conditions.

Seed quality is of increasing importance as a result of climate development. Thus, seed vigor with regard to tolerance to drought and low temperatures as another potentially selectable trait which can be evaluated. The significance of this trait was documented by [17], who reported that an increase in the mean germination time due to poor seed vigor resulted in a significant loss in grain yield. The largest effect was found for winter wheat in which an increase in the mean germination time from 2.1 days to 3.6 days resulted in a relative loss in grain yield of 16%. It is possible to conclude, that increasing the sowing rates of low-vigor seed lots did not secure an optimal grain yield.

High seed quality may be particularly important in low-input agriculture because poor early performance is not as readily compensated for later on by mineral fertilisers and pesticides as it is in conventional agriculture. Furthermore, quality and seed vigor are important factors for competitiveness against weeds: the seeds of low vigor resulted in a perceptible increase in weed biomass and decrease in crop yield.

Various seed germination tests, under the suboptimal conditions of temperature, oxygenation, and water potential of the medium, or undergoing accelerated ageing and controlled deterioration allows for the sensitive differentiation between seed lots. Germination and vigor also depends on multiple biochemical and molecular variables, and its characterization is expected to provide new markers of seed quality that can be used in breeding programmes.

Significant correlations between field emergence and laboratory tests of vigor have been published [18, 19, 20]. Higher precipitation shortly before the harvest decreased the seed vigor of spring barley significantly [21, 22]. Higher air temperatures during this period and during the period April–July increased vigor significantly. Seed germination and vigor were related

to the parameters that are important for malting. The germination capacity of all lines was higher than their vigor and germination energy: 2.9% higher than vigor and 4.6% higher than the germination energy on average. This finding has confirmed the opinions of many authors who have reported that seed performance under optimal conditions is often higher in comparison to the seed performance in vigor experiments under stress and field conditions. Moreover, it has been confirmed that samples of the same germination capacity may have different vigor and storage potential.

The influence of late-terminal drought stress during grain filling on the germination and vigor of barley seeds has been studied [23]. Stress during the grain-filling stage had no effect on the standard germination test, but it obviously decreased the vigor of the seeds. The results indicate the positive influence of high air temperature during ripening and negative influence of high precipitation on the seed vigor.

The seed vigor of soybean, as evaluated as the mean percentage accelerated ageing rate, can be improved by breeding, whereas high yields were maintained because of the predominance of the general combined effects of both the seed vigor and yield [24]. The seed vigor, as evaluated by the cold test, showed estimates of the genetic response to selection in flax [25]. The three key traits of seed vigor in *Brassica oleracea* were rapid germination, rapid initial downward growth of the seedling, and a high potential for upward shoot growth in the soil with increasing impedance. This result suggests a strategy of stress avoidance. In addition, quantitative trait loci (i.e. QTL) were identified for marker and candidate gene identification. A few genomic regions (QTL) were identified for seedling vigor in rice. For these QTLs, significant genotype and environmental temperature interactions were found [26].

Our previous results [21] indicate the possibility of successful selection for higher seed vigor as an important factor of agronomic and malting quality, even in good years (vigor 93–95%), for the traits given above. However, in the years with generally much lower vigor (61–86%), the success could be more responsive because the effect of the variety prevailed over the effect of the environment for bad years. The vigor of 12 combinations from two locations was compared with vigor of their parents. Significant correlation was found between the vigor of the mothers and their progenies ($r = 0.832$; significant on $P \leq 0.01$), between that of the fathers and their progenies ($r = 0.882$; significant on $P \leq 0.01$), and between the vigor of both parents and their progenies ($r = 0.894$; significant on $P \leq 0.01$). This is further evidence for potential effective breeding for vigor.

A lower seed vigor was correlated with a high occurrence of fungi (as indicated by ergosterol assays) and to a lower percentage of field emergence [27]. Vigor was also related to bread quality [28]. Grain samples with 80–90% vigor produced the greatest bread volume. Grain with vigor below or above this range produced less voluminous loafs of bread. High-quality varieties had a higher content of total polyphenols than did varieties of lower quality and the polyphenol content was correlated to vigor.

Cultivars of wheat with enhanced early vigor are still not commercially available.

3.2. Plant roots

Contemporary knowledge confirms the possibility of selection for the root system and stress root tolerance on the basis of seedling stress tolerance, i.e. at time of the sprouting. It is possible also to evaluate characteristics of seeds and seedlings, i.e. provide selection, after plant hybridization of the plants on the basis of the seed and seedling traits, for seed quality and also classic selection in plant breeding.

Plants have developed different root system size (=RSS) during evolution and breeding. In dry and low levels of nutrients in the soil environments a greater RSS is found enabling plants to be more efficient with their use of water and nutrients from lower soil layers. Varieties of cereals with greater RSS better use soil water and nutrients in dry environments than varieties with smaller RSS. Relations between RSS and yield level, variation, and quality should be studied in a broader range of environments in central Europe for agricultural crops. However, yield is a polygenic trait and its level cannot be therefore explained by variation in only one factor (e.g. RSS). The level is limited primarily by the factor in minimum as described by Justus von Liebig. During vegetation, further limits occur either at different or similar times.

The use of natural resources of agricultural and forest ecosystems is much dependent on the functioning of plant roots. These provide several goods and services to society in the forms of, e.g., yield production, carbon sequestration, avoidance of nutrient release from the soil, alleviation of floods, and energy production. The functioning of plant roots is much less well known than that of the shoots, mostly due to methodological reasons. Only special new technology allows us to address the whole root systems quantitatively. Roots are exposed to several stresses (e.g. water stress by drought, soil frost, hypoxia, water shortage by competition) during their lifetime which may decline their capability to provide goods and services. This is especially the case for trees whose lifespan ranges from tens to hundreds of years. Soil conditions will change with climate warming in many locations, linked to a change in precipitation in summer and winter seasons. Therefore, knowledge of the limits for stress tolerance of the roots of herbaceous and woody plants is demanded for future projections.

The “Green Revolution” created dwarf varieties capable of responding to higher fertilizer inputs without lodging, but failed to reach resource-poor farmers. Crossing early green-revolution wheat, with an F_2 of Norin 10 or Brevor, reduced root biomass. Later generation, semi-dwarf wheat showed genetic variation for root biomass, but some generations exhibited a further reduction in root size [29]. Beside a better use of available water resources, an improvement in the uptake efficiency of nutrients from mineral and organic fertilizers would have an important economic and ecological impact for a resource-efficient agricultural strategy. Varieties with greater roots could enable better use of available nutrients and water, as shown e.g. for phosphorus. The selected varieties with greater RSS should be better adapted to soil problems, like lowering of groundwater tables, acidification, loss of organic matter, soil compaction by heavy machines, etc. Varieties with greater RSS could be bred as catch crops or for the phytoremediation of nutrients and heavy metals. Wheat genotypes with superior root characteristics for efficient nutrient uptake, especially during the tillering and booting stages, should be developed in breeding programs to increase grain yield and minimize nitrate leaching [30].

The effect of water and nutrient application on yield has led to the overuse of these practices in the last decades. This misuse of irrigation and fertilizers is no longer sustainable, given the economic and environmental costs. Transpiration stream largely determines the availability of the mineral N in the rhizosphere. This makes our poor estimate of root densities a major obstacle to any precise assessment of nitrogen availability in fertilized crops. A larger investment by the crop in fine roots at depth in the soil, and less proliferation of roots in surface layers, would improve yields by accessing extra resources. The economic return on investment in roots for water capture was twice the investment for nitrogen capture. An early and more extensive horizontal growth of wheat roots in the 0.2–0.7-m layer of the soil profile in glasshouses was found to improve substantially the uptake of N by vigorous lines [31]. There has been a long-standing interest in varietal differences in the uptake of nutrients, especially of N and P, but progress has been slow in translating this into information that can be used in breeding. Root systems limit plant breeding [32].

Breeding for RSS as a strategy for improving yield stability and crop productivity under dry conditions however is still largely ignored in the breeding process, when it is not the breeding aim as such, e.g. for root crops like sugar beet. The main reason for this shortcoming in breeding for drought tolerance is the lack of a suitable method for evaluation of RSS. An improvement in water use is relevant when soil water remains available at maturity or when deep-rooted genotypes access water in the soil profile that is normally unavailable. At moderate drought, productivity of cereals can be improved by a more effective use of available water, i.e. by increasing the plants access to a higher soil volume by a deeper root system and eventually an increased rooting density in deeper soil. Varieties with a deep root system (Fig. 8) should have more opened stomata to cool the plants by transpiration, and therefore improve their tolerance to high temperatures [33].

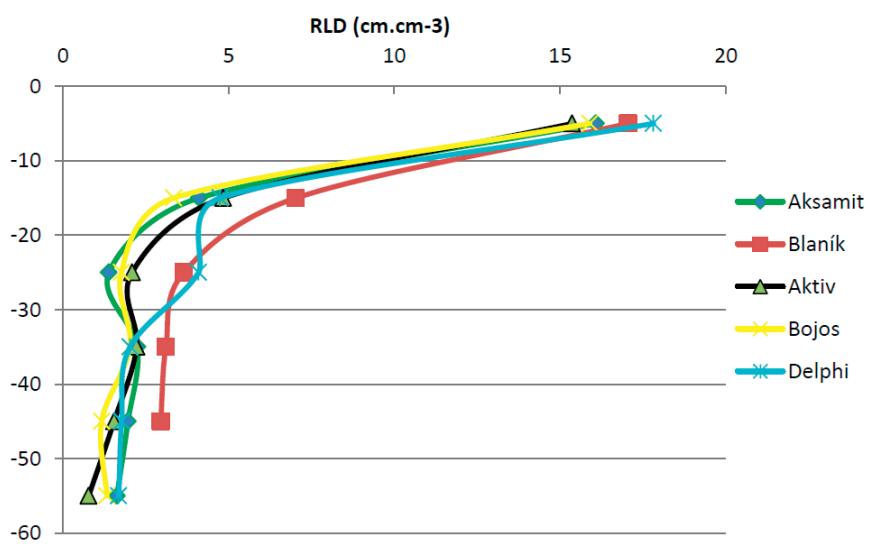


Figure 8. Vertical distribution of root length density (RLD) in spring barley varieties within the soil profile (Hrubčice, Czech Republic, 2012).

Better use of nutrients, including water, due to greater RSS, means:

1. lower the contamination of the environment by them, i.e. lowering the content of nitrates, phosphates, etc. in groundwater, rivers, and seas;
2. a higher adaptability to some stresses during plant growth and development, e.g. limited water, heat, and nutrients;
3. avoidance of water shortage caused by a lowering of the water table in the soil;
4. a better recovery after damage by the limited stresses and after disease and pest attack;
5. higher yields when other growing conditions are not limiting. However, when growing conditions are limiting and water and nutrients are not available, a greater RSS could be a disadvantage;
6. suitability for ecological and/or low-input farming; and
7. lower costs for crop production.

Serious environmental impacts were associated with an enrichment of surface water and groundwater by nitrogen and phosphorus. Increased intensity of livestock rearing depends in Europe in particular on large amounts of imports of nutrients – rich feedstuff from countries outside Europe. In specific areas this has led to nutrient surpluses, which have contributed to problems such as eutrophication.

There are a number of works that assess interspecies or intervariety differences in the above-ground biomass production of crops. Only a small number of authors deal with quantitative and qualitative assessment of underground biomass in relation to the dynamics of nitrogen in soil. An appropriate measure for the use of nitrate nitrogen from the soil in the autumn is the inclusion of cover crops into crop rotation. Field trials [34, 35] were executed to evaluate RSS in eight varieties of white mustard and five varieties of *Phacelia* on two locations, in three BBCH phases (i.e. international scale used to identify the phenological development stages of a plant) over two years. The relationship between RSS, aboveground biomass, and content of nitrogen in the soil was investigated. *Phacelia* featured on average a higher root/shoot ratio (0.45) than mustard (0.32), whereas the year impacted the ratio more than the production area type. In *Phacelia* a highly significant positive correlation was found between aboveground biomass and the amount of soil nitrate nitrogen. This phenomenon confirms that greater biomass production does not mean there is a lower soil nitrate nitrogen. A relationship between the RSS of mustard and the content of NO_3^- and NH_4^+ ions in the soil after harvest was observed. RSS negatively correlated with the content of nitrate nitrogen in the soil, however, the correlation was statistically not significant. Evaluation across sites revealed a positive correlation between aboveground biomass and the amount of residual NO_3^- ions in the soil. On the other hand a significant negative correlation ($r=-0.81$) of RSS and NH_4^+ ions content was observed.

The effect of drought stress on the monitored traits of the root system and aboveground biomass of spring barley (*Hordeum vulgare* L.) was evaluated in a pot experiment by [36]. The characteristics of three varieties in a three-year observation in four different irrigation treatments were evaluated: length, surface area, weight of the root system (evaluated by the soil-core method with subsequent digital image analysis), the RSS (detected by measuring its

electrical capacity), and dry matter yield of aboveground biomass and root biomass – the shoot ratio. Dry matter yield of aboveground biomass significantly correlates with the RSS ($r = 0.700$; significant on $P \leq 0.01$). The variability of root system traits was affected by year (40%–50%), treatment (10%–11%), and variety (8%–14%). Weight ratio of aboveground biomass and root:shoot ratio were affected largely by variant (28.1% and 42.0%; significant effect). Year worked at least root: shoot (15.6%; significant effect). Variants without stress produced the most above ground and below ground biomass. However, the root:shoot ratio was the lowest in this case.

3.3. Examples of effective selection for greater root system

RSS was one of the selection criteria in the breeding for dinitrogen fixation. Breeding of varieties with greater RSS (alfalfa Zuzana), and greater RSS and higher dinitrogen fixation (alfalfa Nitro, white clover Nivel) has been successful [37].

In cereals, root densities of 1.0–1.5 cm cm⁻³ are needed to extract plant available water from the soil, e.g. [38, 39]. Maize plants rarely achieve this below 70 cm, but values of 3–5 cm cm⁻³ or more are common in the top 30 cm of soil. For better exploitation of available water, a better distribution of roots in the soil profile is preferable to partitioning more dry matter to roots [40]. A field trial with selected varieties of spring barley has been realized [36]. For five varieties RSS, its vertical distribution in the soil profile layers up to a 60-cm depth, and grain yield were evaluated. The impact of locality, year, and variety on root system attributes was quantified. Highest values of root length density (RLD) were determined in the layer between 0 cm and 10 cm (Figure 8). A tendency to increase RLD in both research localities and in most varieties in the layer between 40cm and to 60 cm was detected. A significant dependency of grain yield on RLD was only determined in the middle layers of the soil profile. In wet years a significant negative correlation was determined.

The RSS has been found to be a genetic trait, and some specific genes have been observed to control this property. In wild barley, the gene Hsdr4, involved in water-stress tolerance, was located on chromosome 3H near sdw1. This was identified as a marker of QTL for great RSS [41]. Therefore, the RSS and drought tolerance per se maybe linked.

The isolation of intact living root systems from soil in the field has not yet been published and seems impossible. This difficulty is evidenced in many observations. Biomass estimates from minirhizotrons indicate that the <0.25 mm diameter roots (Fig. 9) account for nearly 95% of the total root length [42]. Root separation using a sieve with a 0.5-mm mesh screen led to a marked underestimation of root length density and root biomass. Values up to three times higher were observed when using a 0.2-mm mesh screen in comparison to a 0.5-mm screen [43]. Ex situ methods are expensive and connected with relatively high experimental error. More progressive are in situ methods, in particular use of electric capacitance. Comparison of this method with the ex situ soil-core method found a good correlation. The electric capacitance can be therefore recommended as a quick and cheap method, which enables repeated evaluation of vegetation and retains the evaluated plants until harvest.

The capability “to see” the roots in the soil plays a key role in the evaluation of the potential of herbaceous and woody plants to produce goods and services for society and in the detection of the stress thresholds of roots. From a functional point of view, fine roots (Fig. 9) are the most important for plants. Thus, a method that gives a measure of the root surface area for absorbing water and nutrients would be valuable and it would offer wide applications for users of the natural resources of plant/forest ecosystems.



Figure 9. Measurement of the finest structures of a root system is possible using the method of electrical capacitance.

Wheat (*Triticum aestivum* L.) has been systematically bred for about 200 years. However, this breeding has been done using only aboveground plant parts. We evaluated previously the roots of 18 wheat populations [44]. The RSS was evaluated by its electrical capacitance directly in the field (in situ). The RSS of plants in third and fourth generations were evaluated during shooting and heading. In these evaluations plants were selected for large and small root systems. In dry environments, the progeny of plants with large and small root system had yields of 17.1 and 10.9 grams per plant in the third generation and 18.5 and 10.0 grams per plant in the fourth generation (Fig. 10). The selection process showed a greater response for larger root system size. Selection for higher wheat RSS can be easily used to breed for drought tolerance and higher efficiency of water and fertilizer use.

The study [45] introduces the evaluation of RSS for the breeding of spring barley, in particular for drought tolerance. The aim of this study was to present the method of RSS evaluation and show it in practical use, in particular in relation to drought tolerance. The varieties of spring barley were evaluated for RSS by its electrical capacity. The RSS was compared with the grain yield and grain quality of the varieties at 7–19 stations each year. Varieties with a greater RSS had a significantly higher yield in the dry part of the year. Varieties with a greater RSS had significantly higher contents of starch, saccharide extracts, and malt extracts, as well as higher

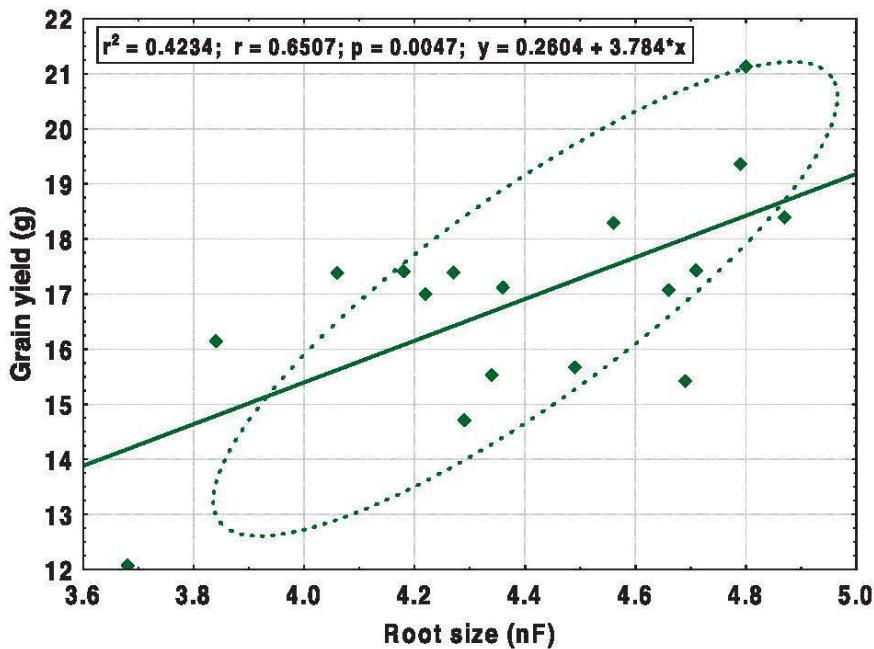


Figure 10. Regression relationship of the wheat grain yield on RSS as averaged from three locations (published in [44]).

yields of protein and starch in dry environments. It can be concluded that a small RSS is related to a low grain yield and malt quality in dry environments, even in genetically diverse varieties.



Figure 11. The root is the most sensitive organ of the plant. On the left: tree sample *Pinus sylvestris* (Scots pine), standing on the main root, on the 4 m height, due to influence of strong soil erosion. These trees have on the basis of measurements at least twice as large a root system compared to those in a conventional environment. In the other two images are oil rape roots. The left of these images is a root located from a relatively dry, well-prepared soil. The right of these images shows a root from compacted, moist soil. Such changes have an effect on the metabolism of plants, yield, seed quality, stress resistance, i.e. not only variety, provenance, and method of seed storage. In addition, the quality of soil preparation (at field crops) has a large influence.

The relevance and response to selection for greater RSS of spring barley in field conditions has been studied [46]. The effect of selection in 12 barley populations developed by mutual crossing

of 4 parents in the F₃ generation as a response to the selection in the preceding generation F₂, for great and small RSS was evaluated. The selection was effective. The average difference in the parental segregating generation was +40 and -43%, and in progeny +4 and -2%, respectively. Grain yield responded two times more to the selection (plants with a root size greater by 3.9% had higher yield of 8.1%). Root size was found to be related to grain yield. Some varieties donated greater, some smaller roots into progeny. It was shown that the selection for RSS was effective and responsive (more for greater than for smaller roots) in a similar way as for grain yield and can be therefore used in practical breeding.

4. Importance of plant integrity in crop research

Plant integrity looks like as a “very easy and expanded topic,” but the reality is very different. “Thanks” to the rigid specialization of scientists, we are losing a holistic view of plants. It is necessary to sense a plant in its entire complexity – both roots and shoots, as well as across their life cycles. Complex research, i.e. the connection of biology, plant nutrition, ecology, and other disciplines, is hardly observable in most scientific work. Only such an integrated approach can allow us to reach the correct interpretations for experimental results [47–54]. For example, when interaction of three or more stressors exists, there can be a lower or higher effect of stressors (compensated through the course of metabolism, etc.). The reason is the influence of individual factors on metabolism and their possible antagonistic influences. Stressors always have pleiotropic effects on a plant, and influence on many genes. Therefore, it is very important to test the adaptability of new breeding materials by utilizing extreme conditions in more locations, such as in ICARDA (The International Center for Agriculture Research in the Dry Areas) and CIMMYT (International Maize and Wheat Improvement Center) in Mexico [54, 55].

Currently, research is booming for the use of model, standard plants (*Arabidopsis* etc.). It is desirable that agricultural research is conducted with crops and not with so-called model species. The reasons for this requirement is both practical and physiological (diversity of metabolism). If, for example, *Arabidopsis* is used as a “model plant” for oilseed rape, because it is (on the basis of a physiological viewpoint) a similar plant with regards to its metabolic functions, then why not start research work straight away with oilseed rape?

There is also other problem. The differences between biological and agricultural research has increased in many cases. This phenomenon can also be observed in the relationship between contemporary agricultural research and its practical applications. Narrow research specialization predominates strongly, and the differences between biological and agricultural research has increased in many research areas. There is a relatively significant lack of new methods for the analysis of this problem. To be honest, lot of different types of internal plant signals, i.e., the transport of information in plant metabolism and their interconnection, memory of plants, and importance of plant neurobiology is also neglected [56].

Productive genotypes have a more efficient metabolic activity throughout all vegetation, with faster spring root growth and better plant cover affecting their subsequent growth and

development. In our experiments, the influence of provenance was greater than that of variety (this is not a rule, it may be the opposite due to differences between varieties and locations at each experiment). Provenance has a very significant influence on the physiological characteristics of seeds, an even greater influence than seed weight and germination. The results confirm the importance of quality seeds to the cultivation of field crops and especially highlights the importance of plant integrity [57–62]. For example, analysis of the effect of variety and provenance of oilseed rape seeds (*Brassica napus L. ssp. oleifera*) on germination under different temperature conditions was provided. The obtained results confirm the importance of seed provenance and emphasize a preference for high-quality seeds. It is known that good seed vigor and provenance guarantees not only better field emergence, growth, and development of overall resistance to stressors (drought, high temperature), but also has a significant impact on seed yield in the subsequent generation (according to better overwintering).

There are several ways to improve the resistance and tolerance (adaptability) of plants to the variable environmental stress conditions with regard to the basis of the whole plant physiology.

The last physiological studies on the rape plant integrity and plasticity have shown, that the crop “responds” to stressors by more than 100 of currently known and measured physiological traits (as mentioned in the previous section of this chapter). There is possibility to use this phenomenon in plant breeding. Selected basic traits of seeds as a vigor, germination percentage, emergence, and stress tolerance of germinated seeds to high and low temperatures has significant influence on the yield of winter oilseed rape varieties [57–63], see below. These problems require more detailed analysis [64–67]. Adaptable varieties have a wider possibility for growth and development in field conditions. Unfortunately, the current system of approving new varieties in many European countries is not heading in the direction of adaptable varieties, but rather in the direction of morphological homogeneity of the genetic material.

4.1. The results of experiments with the oil rapeseeds

Genotype	Country of origin	Company origin
Californium	France	Monsanto SAS
Viking	Germany	NPZ (Norddeutsche Pflanzenzucht)
Navajo	Great Britain	CPB Twyford Ltd
OP 4947/07	Czech Republic	Oseva PRO, s.r.o.
Cadeli	France	Monsanto SAS
Grizzly	France	Société RAGT 2n
ČŽL 20	China	Breeding material
Labrador	France	SCA Adrien Momont et Fils

Table 1. Oil rapeseed cultivars at additional tests

On the basis of laboratory additional tests with the selected cultivars (Tab. 1) the following results supporting the importance of the integrity of plants were obtained:

1. It is possible to eliminate, at the seed level, genotypes (initial breeding materials and cultivars) which do not tolerate extreme temperature and temperature changes during germination, have low water use efficiency, and are intolerant to abiotic stresses.
2. It was confirmed that these genotypes also have poor field emergence and initial growth of roots with implications for a further vegetation period, mainly for overwintering and spring regeneration which has influence on the yield.
3. This test method represents a tool for the screening of genetic resources with resistance to abiotic stress and this technology process is acceptable also for other crops.

Presented results were confirmed in the other rapeseed varieties (36 cultivars). Important obtained relationships are given in Tab. 2.

Seed germination after exposure to cold temperatures for three days after the saturation of seeds by water – simulating the impact of early frosts (20°C/12 hr a day, and -3°C/12 hr a night)	+0.56**
Seed germination after exposure to high temperatures for three days after the saturation of seeds by water – simulating the impact of high temperature (30°C/12 hr a day, and 20°C/12 hr+0.47* a night)	
Influence of the locality (provenance)	+0.78**
Influence of the cultivar	+0.68**
Efficiency of water utilization (water content required for germination) at standard conditions during sprouting	+0.35*
Efficiency of water utilization at high-temperature conditions during sprouting	+0.88**
Efficiency of water utilization at low-temperature conditions during sprouting	+0.25

Note: Stress tolerant seeds at time of sprouting are one of the lot of guarantors of the quality crops establishment during growth and development and for winter survival.

**Statistical significance at 1%; *Statistical significance at 5%

Table 2. Correlation coefficients: seed physiological traits and the yield.

However, one very important problem in plant experiments remains. There is the possibility to select pairs of cultivars with different traits and for simplification from the same latitude it is possible to obtain many conclusions. In this case there is a random drift of results. If we use large groups of cultivars it is possible to obtain, for different physiological types, common and repeatable conclusions.

The cultivars used for scientific experiments are not often appropriate for the analysed target. Shortly: There is in lot of “physiological experiments” with comparison of only two contrasting

genotypes (varieties). It is very little number for finding general dependencies. (Then there plays the role random drift as mentioned). This type of scientific work has both physiological and genetic aspects, logical reasoning during the writing of scientific articles, and is at a high level; we can read a productive scientific article, but the practical application of the results for plant production and breeding then becomes impossible. It is not possible to draw general conclusions based on the analysis of two genotypes especially if whole plant physiology is important to the solution of the problem.

5. Conclusions

It is possible to provide selection for cultivar resistance to stress already at the seed germination stage and on the quality of plant root systems. Quality of the embryonic roots is important for the following growth and root development. Why does this possibility exists? The answer is because in the juvenile phase and in later stages, the same genotype is still active.

The effect of plant integrity and of aboveground–belowground defense signaling on plant resistance against abiotic stresses (and also pathogens) is important and a relatively new subject of scientific research. Biochemical analysis has unequivocally proven that plant defense responses to stress and pathogen infection differ between whole intact plants and detached plant parts (leaves, roots, stems, etc.). Therefore, the question arises – which type of scientific work will reflect reality more realistically? It is possible to conclude that it is research on the basis of plant integrity.

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The Trials and Tribulations of the Plant Male Gametophyte – Understanding Reproductive Stage Stress Tolerance

Ettore Pacini and Rudy Dolferus

Abstract

Yield and productivity of many crop species depend on successful reproductive development to produce seeds or fruits for human nutrition. Plants determine the right time to flower based on environmental cues (day length, temperature) and angiosperms have evolved a plethora of mechanisms to adapt flowering to specific environmental conditions. Despite these adaptation mechanisms, fertilisation and seed production remain subject to the reigning weather conditions before and during flowering. To fertilise the immobile female gametes inside the ovule, the male gametophytes need to be dispersed in a hostile environment. In crop plants, unexpected inclement weather conditions during male gametophyte development and pollen dispersal are often associated with dramatic yield losses. Molecular and physiological studies are gradually making progress in identifying genes and processes that control various aspects of pollen development, but the many intricacies involved in environmental control of pollen development and – in particular – regulation of male fertility remain poorly understood. The aim of this paper is to draw attention to the enormous amount of complexity and biodiversity that exist in angiosperm male gametophyte development. A better understanding of the strategies that exist in adapting pollen production and fertility to environmental challenges may ultimately benefit improvement of abiotic stress tolerance in major food crops.

Keywords: Male gametophyte, pollen, development, abiotic stress, angiosperms, fertility

1. Introduction

The reproductive cycle in plants alternates between a haploid gametophytic and a diploid sporophytic generation. During the evolution from green algae (Charophytes) to land plants, the dominance of the gametophytic generation has gradually decreased in favour of the

sporophytic generation. Originally, the gametophyte and sporophyte were separate independent organisms with very different appearances. In the first non-vascular land plants (liverworts, mosses) the gametophyte was still dominant, but in the first vascular land plants (ferns) the sporophyte prevailed. In ferns, the gametophyte is still an independent organism but with vastly reduced size. In seed-producing higher plants (Spermatophyta: angiosperms, gymnosperms), the gametophyte reduction became extreme (only a few cells) and both male and female gametophytes became physically part of the sporophyte [1, 2]. The emergence of the sporophyte as the dominant phase of the life cycle in seed plants has been attributed to genetic complementation and the capacity of the diploid stage to mask deleterious DNA mutations, an idea that was supported by the fact that land plants had to adapt to a more hostile environment. This argument has been disputed and the exact reason why the diploid sporophyte stage became dominant in land plants is still being debated [1, 3–5].

The ecological pressure to adapt to a dry environment with exposure to many new environmental stresses (water stress, UV light and heat) required a lot of morphological and developmental changes during the evolution from mosses and ferns (Archegoniatae) to Spermatophyta [6]. The generation of the vascular system, roots, stomata and the hormonal system that regulates these developmental features in Spermatophytes evolved along with adaptation to new environmental challenges [7–9]. The next step in the evolution of land plants was the establishment of sexual reproduction in a land environment and the development of gametophytes with different sizes and sexes (heterospory). Sexual reproduction offers an opportunity to recombine combinations of genetic traits and spread genetic variability between populations. This new-found capacity played a major role during evolution in the adaptation of plants to the terrestrial environment [10–12]. Sexual reproduction became therefore the prevalent reproduction system in both plants and animals [13]. The immobility of the sporophyte in land plants makes pollen and seeds the only vector systems to exchange genetic information between plant populations. Pollen production and pollination are critical in the breeding system of land plants, and the large biodiversity that evolved in plant pollination mechanisms illustrates the tight linkage with environmental adaptation [11, 14].

The origin of pollen can be traced back to heterosporous Pteridophyta (vascular plants) [15–17], which have microspores with features that are reminiscent of pollen: similar cell wall (intine and exine), storage reserves for the first stages of growth, reduction or absence of watery vacuoles at maturity [18]. In seed plants, the female gametophyte is immobile and develops totally inside the ovule of the ovary [19]. This makes pollen grains a crucial mobile vector for exchanging genetic information between different plant populations. The male gametophytes form inside the pollen sac in gymnosperms and in the anthers of angiosperm flowers (Figure 1) [2, 20, 21]. Pollen grains need to be dispersed from the anther and travel to the stigma to fertilise the immobile egg cell inside the ovule(s) of the ovary. This ovary can be located in the same flower, another flower of the same plant, a neighbouring plant or a more remote plant. The tough multi-layered pollen wall is an adaptation to protect the male gametes against environmental stresses during presentation and dispersal, while it is at the same time adapted for different pollen dispersal methods [22, 23]. The pollen dispersal methods and breeding systems in plants are amazingly diverse [14, 24]. Following domestication, many crop species

are grown in environments that are vastly different from their original growth habitat. The breeding system of many crop species may therefore not be optimal for their current growth habitat, let alone whether it will be adapted to a future world with a different climate. In many staple crops (e.g. rice and wheat) male reproductive development is considered the 'Achilles tendon' of reproductive development, with massive yield losses under unexpected adverse weather conditions (heat, drought, cold) becoming increasingly common occurrences [25–28]. Although the generation of haploid male gametes in angiosperms occurs via a conserved pathway, there are many variations present in different plant species in the way this process proceeds. In many cases, this biodiversity can be associated with adaptations to particular environmental restraints. This paper will explore the complexity in angiosperm pollen development and investigate how it can contribute to a better understanding of abiotic stress tolerance of male reproductive development. The focus of this review paper will be on the interaction between environment and pollen developmental processes and not on the diversity that exists in pollen–stigma compatibility and plant breeding systems. A supplementary glossary of commonly used terms and definitions related to male gametophyte development is supplied for those readers who are less familiar with this subject (See Appendix).

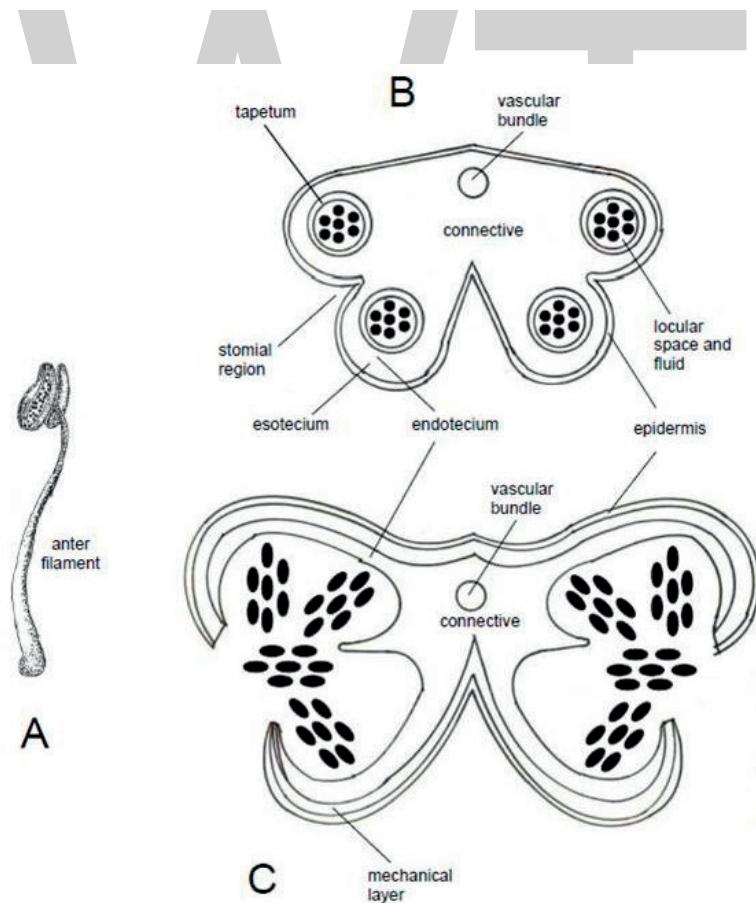


Figure 1. Schematic drawing of an ideal stamen (A), anther at microspore stage (B) and just after anther opening (C) with their components and functions. Water and some nutrients are transported by the vascular bundle from the mother plant via the filament towards the anther. Nutrients move to the tapetum via the connective tissue and components synthesised by the tapetum are then released into the loculus, where they are absorbed by the developing grains, and they are either utilised immediately or stored temporarily in the locular fluid, vacuole or amyloplasts.

2. Male gametophyte development and its biodiversity

In angiosperms, male gametophytes develop in the anther. Each anther consists of two thecae, each consisting of two adjacent microsporangia that are separated by the connective tissue (Figure 1). The first phase in pollen development, the meiotic division of the sporophytic meiocytes of the four microsporangia to form haploid tetrads and young microspores, is called microsporogenesis (Figure 2). During the second phase, microgametogenesis, the microspores enlarge and become vacuolated. Vacuolisation and the cytoskeleton force the nucleus to migrate to a peripheral position. The first mitotic division is asymmetric and produces a germ cell that is engulfed by the cytoplasm of the vegetative cell to become physically isolated from the vegetative cell (bi-cellular pollen; cell-within-a-cell). The germ cell then undergoes a second mitotic division to produce the two sperm cells (Figure 2). During fertilisation, one male gamete fuses with the egg cell and the other with the two polar nuclei of the central cell to form the zygote and endosperm, respectively. The male sperm cells are very diminutive in size, but transcriptome analysis has recently revealed that their gene expression pattern is unlike any other plant tissue, suggesting that they are functionally very specialised [29].

Pollen type	Starch content	Two-celled	Three-celled
Orthodox (>20% water)	Starchy	<ul style="list-style-type: none"> • <i>Olea europaea</i> (Oleaceae) PK • <i>Erica arborea</i> (Ericaceae) 	<ul style="list-style-type: none"> • <i>Wolfia arrhiza</i> (Araceae) (PK) • <i>Lilium bienne</i> (Liliaceae) (PK) • <i>Nelumbo nucifera</i> (Nelumbonaceae) (PK)
	Starchless	<ul style="list-style-type: none"> • Solanaceae (PK presence depends on pollination syndrome) • Lamiaceae (PK) • Myrtaceae (PK) <ul style="list-style-type: none"> • Scrophulariaceae (PK) • <i>Acanthus mollis</i> (Acanthaceae) (PK) • <i>Bryonia dioica</i> (Cucurbitaceae) (PK) • <i>Cucumis melo</i> (Cucurbitaceae) (PK) • Liliaceae (some species) (PK) 	<ul style="list-style-type: none"> • <i>Hedera helix</i> (Araliaceae) (PK) • <i>Borago officinalis</i> (Boraginaceae) (PK) • Caprifoliaceae (PK) • Asteraceae (PK) • <i>Canna indica</i> (Cannaceae) (PK) • <i>Tulipa gesneriana</i> (Liliaceae) (PK)
Recalcitrant (<20% water)	Starchy	<ul style="list-style-type: none"> • <i>Cucurbita pepo</i> (Cucurbitaceae) (PK) • <i>Plantago</i> sp. (PK) • <i>Portulaca tuberosa</i> (PK) 	<ul style="list-style-type: none"> • Amaranthaceae (PK) • Alismataceae (PK) • Poaceae
	Starchless	<ul style="list-style-type: none"> • <i>Laurus nobilis</i> PK • Malvaceae PK 	<ul style="list-style-type: none"> • <i>Cereus</i> sp. (Cactaceae) (PK) • Caryophyllaceae (PK)

Table 1. Classification of pollen diversity according to cytological events during pollen development, and examples of some representative plant species. Pollenkitt (PK) is typically present in zoophilous and entomophilous species and is generally absent in anemophilous species, with the exception of *Olea europaea*, a secondary anemophilous species. Some plant families (e.g. Liliaceae) have a majority of members with two-celled starchless grains and some species with three-celled starchless pollen grains [30, 185] (E. Pacini, personal observations).

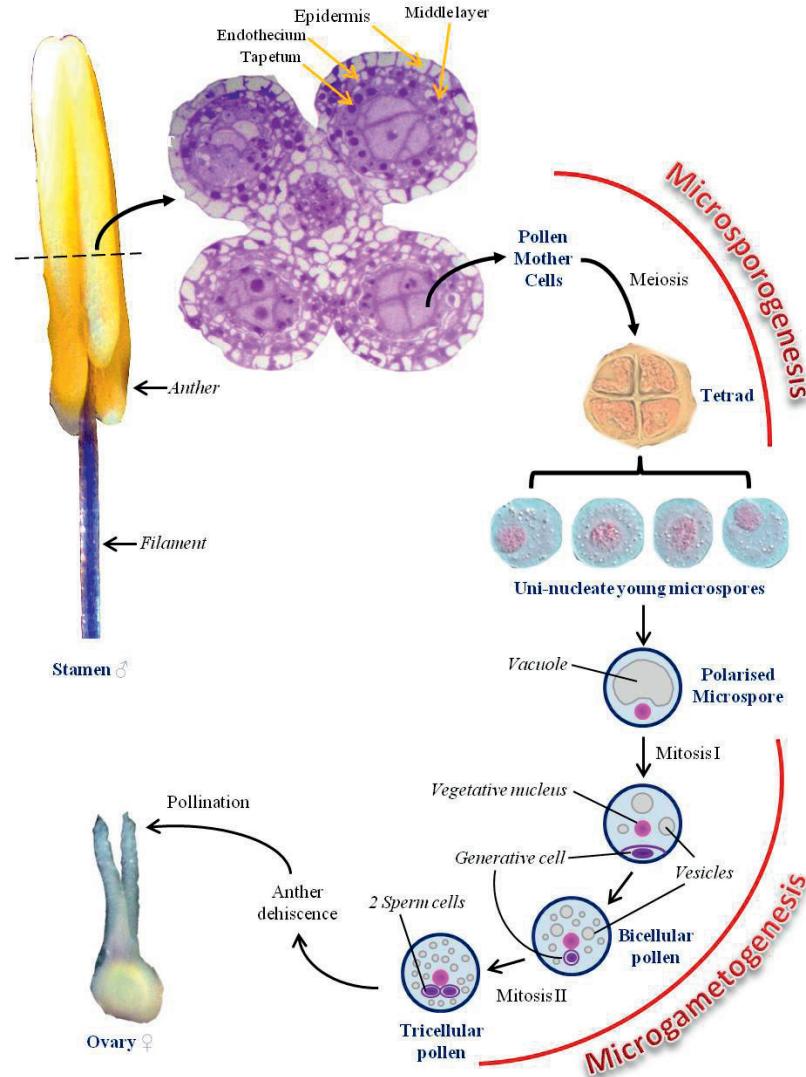


Figure 2. Male gametophyte development in angiosperms. Pollen grains develop in the stamen, which consists of a filament supporting the anther. The vascular bundles in the filament conduct nutrients from the mother plant to the anther. The cross-section of the anther (rice) before the onset of meiosis shows the four microsporangia where the male gametophytes develop. From outside to inside, the anther wall consists of the epidermis, the endothecium, the middle layer and the tapetum. Both the middle layer and the tapetum degenerate towards pollen maturity, leaving only the epidermis and the endothecium to protect the pollen grains in the loculus before anther opening. The central cells of the anther, the pollen mother cells (meiocytes), differentiate and become selectively isolated from the mother plant through callose secretion by the meiocyte cytoplasm. The pollen mother cells undergo meiosis to form tetrads. The uni-nucleate young microspores are released from the tetrad with the help of enzymes secreted by the tapetum [313]. Exine is completed with the intervention of polymers secreted by the tapetum in the loculus [35]. Young microspores have a central nucleus and in Poaceae they are with the pore attached to tapetum until anther opening. The germination pore becomes visible and a large vacuole forms, pushing, with the intervention of the cytoskeleton, the nucleus in a peripheral position (polarised microspore stage) [176, 314]. At the vacuolated stage, the microspores undergo an asymmetric division (pollen mitosis I) to produce the vegetative and generative nucleus. The generative nucleus is then isolated in a separate compartment within the vegetative cell to form a bi-cellular pollen grain (cell-within-a-cell). During pollen maturation, the vacuole of the vegetative cell gradually decreases in size and accumulation of starch granules is observed (engorgement). In plants with tri-cellular pollen, a second mitotic division of the germ cell takes place before anthesis (pollen mitosis II) to produce the two sperm cells. At this stage, the two germ cells are found in close proximity of the vegetative nucleus (male germ unit).

Nuclei number (meiosis, tetrad, microspores), pollen grain cell number (bi-cellular and tri-cellular pollen) and other cytological events (vacuolisation, starch accumulation/hydrolysis, water content) are used to determine pollen developmental stages (Figure 2). These parameters can differ between plant species and differences in pollen development can be used for systematic classifications (Table 1). At dispersal, angiosperm pollen grains can be bi-cellular or tri-cellular (Table 2) [30]. In tri-cellular pollen, the second mitotic division occurs prior to dispersal and pollen is dispersed with the two sperm cells already formed (Figure 2). In bicellular pollen, the second mitotic division occurs during pollen tube growth inside the style-stigma. The term male germ unit describes the relative position and cytological connections between the generative cell, the sperm cells and the vegetative cell nucleus in the mature pollen and pollen tube [2, 31]. Very few species release bi- and tri-cellular pollen grains at the same time. When this occurs (e.g. *Annona cherimola*), the ratio between bi-cellular and tri-cellular pollen grains was shown to depend on environmental factors such as temperature regime and relative humidity during the last phases of maturation [32]. Tri-cellular pollen grains have completed their development before dispersal and are typical for plant families that include important dicot and monocot crop species such as Asteraceae, Lamiaceae, Brassicaceae and Poaceae (Table 1). In some plants, pollen is dispersed as aggregates containing a high number of pollen grains (e.g. massulate orchids) [33]. Orchids are monocots that produce bicellular pollen; the generative cell is spherical at dispersal but changes to the normal spindle shape prior to the second mitotic division when pollen lands on the stigma and starts emitting the pollen tube [34]. Pollen development is further subdivided in early, middle and late stages according to cytological and morphological features such as the presence of a vacuole (Table 1; Figure 2) [35–37]. Vacuolisation occurs only once in some species, but twice in others (once during the early microspore to bi-cellular stage and once during early bicellular to late microspore stage) [18]. Stages of pollen vacuolisation alternate with stages of starch accumulation in plastids (engorgement) and starch accumulation can therefore also occur once or twice. Mature pollen grains can be starchy or starch-less depending on whether starch is present in mature exposed pollen grains (Table 1). Another classification is based on water content of pollen at dispersal: orthodox and recalcitrant pollen is dispersed in partially desiccated or partially hydrated form, respectively. Other differences concern the presence or absence of pollenkitt that distinguish animal/ insect from wind pollinators, respectively (with rare exceptions; Table 1). The diversity in pollen development between different plant species is complex and is functionally important. Different mechanisms have evolved under a variety of environmental constraints to secure pollination success and survival of the species.

3. Meiosis: The start of reproductive development

The decision to flower in higher plants is carefully controlled by environmental stimuli such as temperature and photoperiod [38–42]. After floral meristem initiation and formation of flower buds, meiosis is the committed step for sexual reproduction and formation of the gametophytes. The onset of meiosis is regulated by signals coming from the mother plant. Sugar availability plays an important role in driving cell division by inducing expression of

<i>Locular space availability</i>	<i>Pollen number</i>	<i>Pollen density and dispersal unit</i>
Abundant, space between pollen	Few pollen/locule	6–12 per loculus cross section (Poaceae)
	Many pollen/locule	15–30 per loculus cross section (Solanaceae, Fabaceae, Liliaceae)
Reduced, closely packed pollen	Septate anthers	Compound pollen in each septum, polyad type (8–32 pollen) (Mimosaceae, some Annonaceae)
	Aseptate anthers	Monad pollen, tightly packed, tetrahedral shape (Myrtaceae)
		Compound pollen, very high pollen number, reduced size (Orchidaceae, Asclepiadaceae)

Table 2. Table showing the presence and abundance of locular space and fluid and relationship to pollen dispersal units in angiosperms. The locular fluid volume is extremely reduced when the pollen dispersal unit is of the compound type. Locular space and fluid are present from meiotic prophase until anther desiccation and opening.

the cell cycle regulatory protein cyclin that induces meiosis [43–46]. The initiation of meiosis to form the male and female gametophytes in the anther and ovary is normally a synchronised process [47–52]. However, this is not always the case in some plants and abiotic stresses can cause asynchrony between male and female meiosis [47, 51]. Most commonly, in aseptate anthers all sporogenous initials will proceed to undergo meiosis, while in septate anthers only some initials will undergo meiotic division [53]. This difference will affect locular space and liquid volume available to pollen, pollen number per locule and ultimately the dispersal unit (Table 2). After meiosis, male and female gametophytes follow a very different path of development. While ovule development and maturation is a gradual process, formation of large amounts of pollen grains in the anthers is energetically more demanding. At the time of meiosis, the anthers represent the highest sink strength in the flower and anthers are known to have the highest soluble sugar content of any plant tissue [54, 55]. Synchrony of male meiosis can also be affected in interspecific hybrids [50]. Pollen sterility caused by meiotic asynchrony is a major problem in interspecific rice hybrids where productivity is affected [56]. Mutagenesis approaches in model plants are gradually revealing genes that are involved in initiating meiosis and its progression through the different phases [57–61]. Silencing of the anther-specific zinc finger transcription factor *MEZ1* causes abnormal meiosis and pollen abortion in petunia [62]. The *Arabidopsis STUD, TAM, DUET, MALE MEIOTIC DEATH1, AtKIN14a, b* and *TETRASPORE* genes are responsible for different aspects of male meiosis, such as maintaining pace, synchrony, chromosome organisation and transition between different stages [63–68].

Pollen biotechnology is a potentially powerful tool for crop breeding. Genes that regulate progression and synchrony of pollen meiosis and their regulation (e.g. effect of abiotic stresses) can be exploited for establishing hybrid breeding technologies, for instance, using mutant lines that are conditionally arrested at pollen meiosis [69, 70]. Progress in understanding pollen meiosis will be accelerated by more refined technologies that make it possible to study the meiotic transcriptome in detail [71]. Transcriptome profiling has been used to investigate the

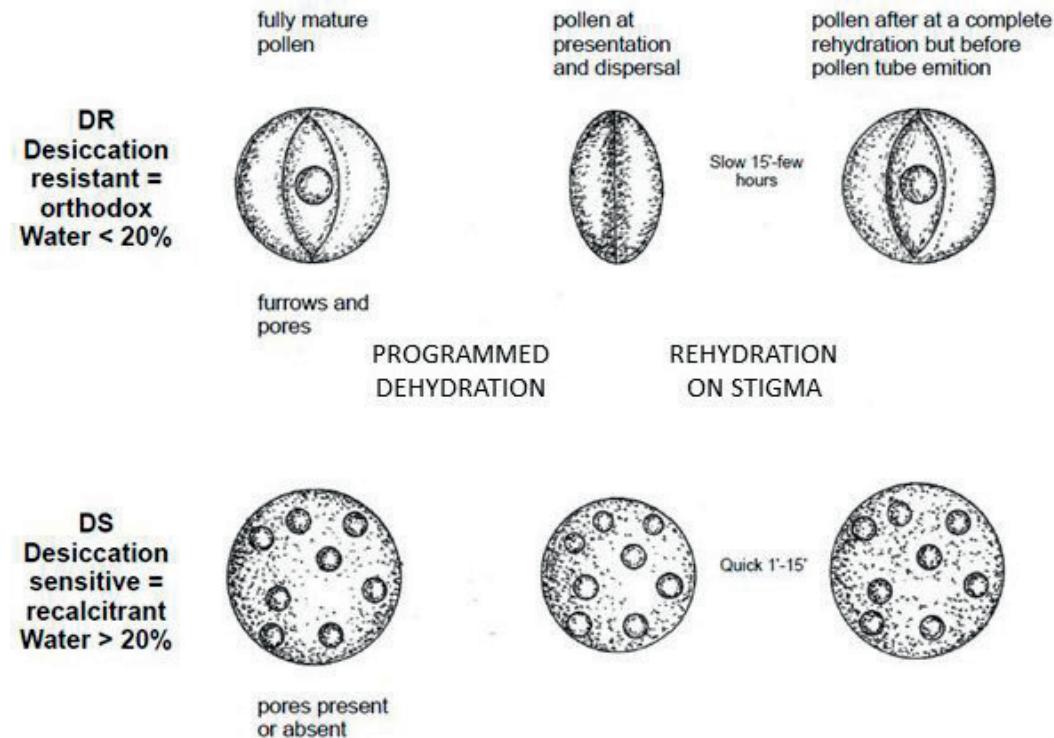


Figure 4. Change of shape and size of pollen according to their water content during the more critical phases of dehydration, presentation, dispersal and rehydration. High temperature and low relative humidity affect desiccation-sensitive pollen (DS) more than desiccation-resistant pollen (DR).

effect of abiotic stresses on pollen meiosis and pollen development [72, 73]. Abiotic stresses such as cold during meiosis can lead to formation of diploid gametes [74]. Polyploidisation and manipulation of chromosome number during meiosis can be used to increase diversity in breeding of crop plants [75, 76]. Some *Arabidopsis* mutants (*DIF1*, *TETRASPORE*, *PARALLEL SPINDLE1* and *Jason*) that affect ploidy levels can improve our understanding of pollen meiosis and how it is affected by the environment [66, 77–80].

4. The importance of the anther tapetum

The tapetum surrounds the pollen mother cells before meiosis and is the inner cell layer of the anther wall (Figure 2). The tapetum plays an important role in pollen development: it secretes the locular fluid containing nutrients for pollen development and deposits components of the pollen cell wall. When these functions are fulfilled, the tapetum undergoes a natural programmed cell death response (PCD) [81–83]. This process is essential to sustain pollen development: PCD generates nutrients for the locular fluid to feed the native pollen grains [81, 83–88]. Tapetal cells are generally polyploid and/or multi-nucleate and are metabolically very active. Tapetal-specific gene transcripts are the most prevalent fraction of total anther transcripts [89]. Polyploidisation and genome endo-duplication are commonly observed in plant tissues with high metabolic activity [90]. High

Pollen size	Water content Shape (dehydrated) Examples	Time for pollen rehydration and tube emission ⁽¹⁾	Consequences
	[H ₂ O]<20% (Orthodox) 	30 min to 1 hour	<ul style="list-style-type: none"> Rehydration and pollen tube emission in extra-stigmatic sites of the flower or other plant parts may occur when air relative humidity rises
15–30 µm	Boraginaceae, e.g. Forget-me-not		
	[H ₂ O] <20% (Recalcitrant) 	From a few seconds to a few minutes	<ul style="list-style-type: none"> Time for rehydration and germination is less for recalcitrant pollen and depends on water percentage during stigma adhesion Due to their small size they lose water quickly and die
30–100 µm (most common)	Spinach, Parietaria, Urtica		
	[H ₂ O]<20% (Orthodox) 	More than 1 hour	<ul style="list-style-type: none"> Orthodox grains of this size resist thermal stress better during presentation and dispersal than smaller orthodox pollen The time for rehydration and germination is also higher compared to smaller orthodox pollen
100–200 µm	Fabaceae, Solanaceae, Liliaceae	A few minutes	
	[H ₂ O]<20% (Recalcitrant) 		<ul style="list-style-type: none"> This category resists thermal and low relative humidity stresses better because of their larger size
	Pumpkin, maize		

⁽¹⁾: Time depends on the status of pollen at stigma adhesion.

Table 6. Table showing the main categories of monad pollen, their size, shape at dispersal, time for rehydration and germination of orthodox (oval) and recalcitrant (spherical) pollen grains, including representative examples and some ecological consequences. The average pollen diameter is 30–100 micrometers with low water content. Orthodox and recalcitrant grains have ecological devices to reduce water loss during presentation and dispersal, e.g. pollen presentation by anthers that are enclosed by the flower corolla and exposing anthers outside the flower as for poricidal anthers.

metabolic activity of tapetum cells is required during meiosis for production of callose, a temporary cell wall that separates the microspores from the tetrad, and for biosynthesis and secretion of sporopollenin for the exine pollen cell wall [91–93]. Mutations that affect callose deposition and dissolution affect microspore development and fertility [94, 95]. The main tapetum nutritional activity occurs during the microspore stage and the first signs of

degeneration do not occur at the same stage in different species [96] but degeneration normally reaches completion near the end of the uni-nucleate microspore stage [97].

The secretory tapetum is the most common type [97]. The tapetum cells form the inner lining of the loculus and remain in place until they degenerate. In some plant species (e.g. Poaceae), the young microspores are found to attach themselves to the tapetum inner wall [35, 98]. In the secretory tapetum, the inner cell wall directed towards the loculus and the radial walls dissolve using a natural protoplasting event to facilitate the secretory function. Orbicules or Uebisch bodies are secreted towards the loculus by the tapetum cytoplasm; their function is not yet elucidated and only unproven hypotheses as to their role have been put forward [99–101]. During development, microspores are dispersed in the locular fluid, the volume of which can vary widely according to anther morphology (aseptate or septate) and the type of pollen dispersal units: more locular fluid is generally present in aseptate anthers and/or when pollen are dispersed as single units, while less fluid is present when pollen are dispersed as aggregates (Table 2) [82]. When released from the tetrad, pollen grains are in direct contact with the secretory tapetum [82]. The substances that are secreted in the locular fluid are neutral polysaccharides, pectins, proteins and lipids, and their relative proportion varies during pollen development [102]. The amount of locular fluid secreted depends also on the number, size and shape of the pollen grains and the dispersal unit (monads *vs.* polyads; Table 2).

Another form of tapetum is the amoeboid or periplasmoidal tapetum which is, for example, found in the Asteraceae family [82]. In this case, the tapetum cell layer undergoes a reorganisation rather than degeneration during its early development. During meiosis, the tapetal cells form long extensions that engulf individual pollen mother cells. At the tetrad stage, the tapetum reorganises to form a periplasmodium which separates the individual young microspores and encloses them within a vacuole in the tapetal cytoplasm [103]. The amoeboid tapetum, better than the more common secretory type, illustrates the nurturing function of the tapetum.

The tapetum forms the interface between the sporophyte and the male gametophyte and is therefore in a strategic position to control reproductive development. Some of the substances entering the tapetum come from the external cell layers of the anther and other parts of the mother plant [104]. The mother plant supplies nutrients via the vascular bundle of the anther filament [84, 105]. Downloading occurs in the anther connective tissue cells and transport to the middle layer occurs symplastically [83]. The outer anther wall cells are connected via plasmodesmata, but the tapetum layer is symplastically isolated from other anther wall cells. Delivery of sugars into the tapetum requires apoplastic transport [55, 106, 107]. The apoplastic cell wall invertase gene is expressed in the tapetum and is responsible for mobilising sucrose into the tapetum cells [108, 109]. Repression of tapetal cell wall invertase activity and gene expression by different abiotic stresses blocks sugar transport to the pollen grains [108–112]. At least in some species nutritive substances are stored temporarily in the tapetum and are then absorbed by the developing pollen grains [102, 113].

The meiotic stage of pollen development is very sensitive to cold, heat and drought stress (Table 3) [25, 28, 109, 110, 114, 115]. It is likely that abiotic stresses at the time when the tapetum is metabolically most active interfere with the synthesis of pollen cell wall components and

the secretion of the locular fluid. This may cause abortion of the young microspores. The formation of the locular fluid is associated with an increase in pollen volume and increased vacuolisation, a process that is affected by water stress (Table 3) [28]. The presence of abundant locular fluid (e.g. Solanaceae and Poaceae) or its extreme reduction (e.g. some orchids, Fabaceae and Myrtaceae; Table 2) has so far not been correlated with higher or lower tolerance to drought stress. Plant species with a periplasmoidal tapetum have a reduced volume of locular fluid. In this case, each microspore is engulfed in the tapetum cytoplasm, so pollen nutrition is direct and does not require an abundant locular fluid [82]. Abiotic stresses may interfere with tapetal PCD and affect its functionality [87]. Both premature and retarded degeneration of the tapetum cause pollen sterility [83, 87, 116–119]. Production of reactive oxygen species (ROS) has recently been implicated in the regulation of PCD timing in the tapetum [120]. ROS are produced in response to many abiotic stresses [121]. Premature tapetum degeneration is a major cause of pollen sterility and yield loss under abiotic stress conditions [118, 122–125]. Carbohydrate mobilisation to the tapetum and its genetic control may play an important role in guaranteeing pollen development under stress conditions. Anther sink strength is reduced in stress-sensitive species [108–110, 126]. At the same time, sugars appear to be redirected to other tissues, e.g. leading to starch accumulation in the endothecium layer of the anther wall [106, 107, 127]. The tapetum is a sporophytic tissue and its function is controlled by signals from the sporophyte (sugars, hormones). Improvement of stress tolerance in crop species will therefore require a better understanding of the effect of stress on the sporophyte, as well as on sporophyte–gametophyte communication.

<i>Stages</i>	<i>Stress type</i>	<i>Targeted stage and/or compartment</i>	<i>Defence mechanisms</i>
Pollen meiosis & further development	· Water stress	· Tapetum, locular fluid formation	· Locular volume reduction
	· Water stress	· Microspore or bicellular pollen vacuolisation	· Anthers protected inside thick flower whorls
	· Low temperatures >0°C	· Cytoplasm activity and cyclosis	· Programmed developmental arrest
	· Heat stress >30°C	· Cytoplasm activity and cyclosis	· Programmed developmental arrest
Anther desiccation	· Rain	· Locular fluid fails to evaporate, anthers fail to dehisce	· Anthers are protected inside the corolla where pollen is exposed
	· Heat stress	· Carbohydrate metabolism	· Synthesis of heat shock proteins
	· Low temperatures	· Carbohydrate metabolism, cyclosis	· Presence of high amounts of LMW carbohydrate reserves
Pollen presentation	· Heat stress	· Pollen water content	· Pollen is presented inside the corolla
	· High/low relative humidity	· Pollen water content	· Pollen is presented inside the corolla and for a short time lapse
Pollen dispersal	· Heat stress	· Number of viable, dispersed grains	· Social plants with shorter pollen flight

<i>Stages</i>	<i>Stress type</i>	<i>Targeted stage and/or compartment</i>	<i>Defence mechanisms</i>
	· High/low relative humidity	· Number of viable, dispersed grains	· Anthers can delay pollen dispersal

Table 3. Effect of abiotic stresses on different stages of pollen development, and possible evolutionary defence mechanisms of the male gametophyte.

5. The pollen cell wall

5.1. Exine

The synthesis of the pollen cell wall starts during meiosis and depends on the activity of the tapetum. The composition of the pollen wall is unique compared to other plant cell walls and shows species-specific diversity. The biodiversity in pollen cell walls is functionally important for the plant to distinguish its own pollen from that of other plants [128–131]. The pollen cell wall can vary physically and chemically to match environmental aspects of pollination. Pollen wall diversity serves a taxonomical value, forming the basis of palynology [132]. The extremely resistant and elastic outer exine wall has evolved to protect pollen during dispersal. Exine is deposited first to provide pollen grains with their distinctive and characteristic features (Figure 3A). Pollen cell wall organisation starts just before meiosis when meiocytes become surrounded by callose secreted by the tapetum [92, 93, 133]. The callose special cell wall (SCW) is formed during prophase and interphase and closes the cytomictic channels that synchronise the first meiotic division. Exine is patterned under the callose layer and the microspore plasma membrane (primexine or exine precursor) at the end of meiosis (late tetrad stage) and is completed after the release of the microspores from the SCW at the end of the tetrad stage. The tapetum then produces callase, a β -1,3-D-glucanase enzyme responsible for dissolution of the callose wall, as well as sporopollenin precursors, a complex polymer of fatty acids and phenolic compounds. These are released in the loculus and polymerise on the primexine of the microspore following its release from the tetrad [132, 134, 135]. Mutant screens for impaired pollen walls in *Arabidopsis* revealed several genes involved in sporopollenin biosynthesis and most of these mutants are male sterile [92, 136–140]. Sporopollenin precursors are deposited by ABC transporters that are expressed in the tapetum at the early vacuolated microspore stage [134, 141]. Sporopollenin biosynthetic enzymes form a complex ('metabolon') in the endoplasmatic reticulum of the tapetum [142]. Recent ultrastructural studies reveal the involvement of specialised tapetum organelles, elaioplasts or tapetosomes, in exine wall deposition [140, 143]. Exine deposition is reduced, interrupted and can even be absent altogether in aquatic plants or plants living and pollinating in extremely wet environments [144]. The absence of exine in species having underwater pollination (e.g., seagrasses) is correlated with the fact that in water pollen grains do not undergo desiccation and have to remain hydrophilic; there is no developmental arrest and changes in shape and volume do not occur [145].

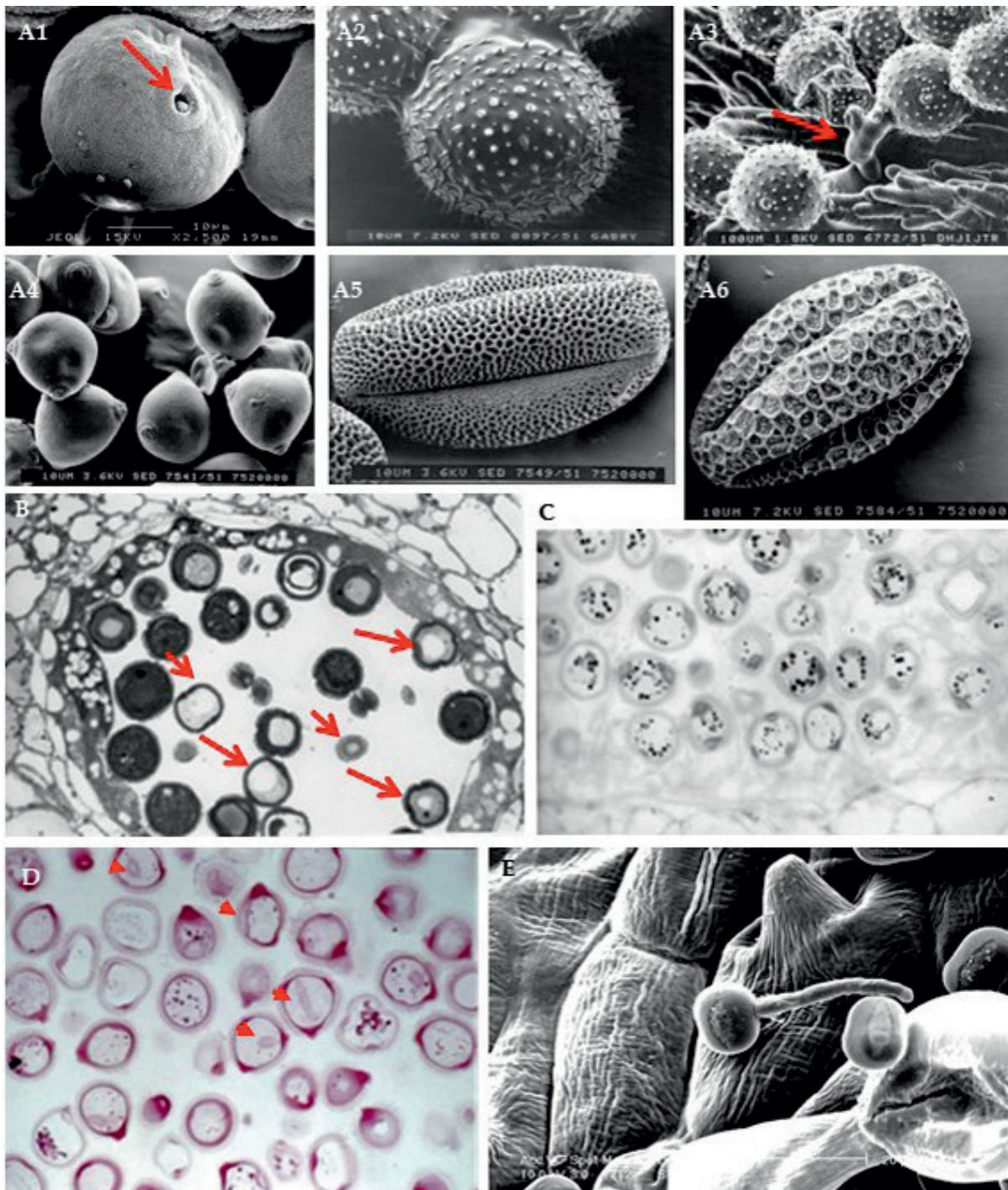


Figure 3. Different stages of pollen development in angiosperms.

A1-4: Scanning electron micrographs of mature desiccation-sensitive recalcitrant grains which are devoid of furrows.

A1: A rice pollen grain close to anthesis, showing the cell wall surface and the germination pore (arrow). A2: three pollen grains of *Lavatera arborea* (Malvaceae) kept together by pollen kitt, a viscous fluid covering the pores of the grains. A3-6: Pollen grains of different members of

the Cucurbitaceae family with recalcitrant pollen grains. A3: *Cucurbita pepo*, with a germinating pollen grain (arrow) taken 10' after pollination. A4: *Cucumis sativus*, a species with recalcitrant pollen grains. A5 and A6: *Bryonia dioica* and *Cyrtellus lanatus* orthodox pollen grains with furrows.

B-E: Asynchrony in vacuolisation and starch storage in olive (*Olea europaea*) pollen grains. B: A section of anther at mid-bi-cellular stage during the second vacuolisation, with degenerating tapetum (toluidine blue O staining). The asynchrony of development of pollen is evident: grains have vacuoles of different sizes and some grains are degenerating (arrows). C: A section of an anther at mid-microspore stage at the first starch engorgement (stained with Periodic Acid Schiff). Starch grains have different sizes because of the asynchrony of starch storage. D: Section of an anther at the early bi-cellular stage and second starch engorgement (PAS stained). Grains have an asynchronous development with respect to starch engorgement and in some grains the generative cell (arrow heads) can be discerned because of the thin polysaccharide wall. E: Pollen grains of *Cerinthe major* (Boraginaceae) displaced by flower visitors on the corolla (SEM). Only one has emitted a pollen tube because of precocious rehydration due to high humidity during the night – probably indicating asynchroneous development of the grains.

5.2. Intine

The exine wall is completed by the mid-microspore stage before the internal intine layer is deposited. Intine is less elastic and consists of a pecto-cellulose mixture. Intine synthesis also starts before the first mitotic division and is always completed by the time the vegetative and generative cells are formed [132, 146]. Mutagenesis approaches have identified genes involved in the biosynthesis of pectins for the intine cell wall [147–152]. Some of these genes are expressed in the tapetum and ABC transporters transfer intine components to the pollen grains [153]. Mutations affect pollen shape and fertility, as well as growth of the pollen tube. Pectin is the main component of intine and is secreted by the tapetum into the locular fluid. Accumulation is highest at the vacuolated microspore stage [146]. The *Brassica campestris* Male Fertility2 and 9 (*BcMF2, 9*) genes encode novel polygalacturonase enzymes that play a role in pectin metabolism, intine formation and tapetum degradation [151, 152]. At pollen germination, the intine wall forms a continuum with the the pollen tube pectocellulose wall.

5.3. The role of the cell wall in regulating pollen size and shape

The pollen wall controls homeostasis of the cytoplasm and reduces fluctuations in pollen volume due to variations in water content. This is important during dispersal, when pollen is exposed to air. The characteristic exine wall furrows and surface pattern are crucial for the harmomegathic functions that regulate pollen shape during dehydration [154] (Figure 3 A5–6). After landing on the stigma, the pollen wall controls the rehydration process with water coming from the stigma in angiosperms or from the ovule in gymnosperms (pollination drop) [155].

The exine layer has generally one or more pores through which the pollen tube is emitted (Figure 3 A1, 3). When pollen pores are absent, the pollen tube is emitted at the site where the

pollen grain contacts the stigma surface. The pattern and distribution of the apertures are determined by the tetrad shape and callose deposition at the intersporal walls [156, 157]. The *Arabidopsis tam* mutant (*tardy asynchronous meiosis*) shows an altered cytoplasmic partitioning (cytokinesis) during tetrad formation and altered aperture patterning, suggesting that the last contact points between the cytoplasms of the future microspores during cytokinesis are the place where apertures are formed [158]. The number of pores per pollen grain can vary within one species and germination speed is positively correlated with pore number [159] and pollen water content at dispersal [160]. The intine wall is a continuous layer but is generally thicker and more elaborate at the pores and/or furrows to support the harmomegathic process [161]. Exine and intine have a similar thickness but in some cases intine, especially in the poral region, is much thicker and very pectin-rich, which may help in keeping pollen cytoplasm hydrated during dispersal [162–164].

5.4. Pollenkitt

In some plant species, the surface of the pollen wall contains various amounts of pollenkitt, a viscous hydrophobic substance. The sticky nature of pollenkitt is thought to play a role in pollen adhesion to pollinators during dispersal [165, 166], but several other functions have been suggested [167]. Plants with zoophilous or entomophilous pollination, some of which having secondary anemophylous pollination, have exine cavities or ornamenations containing pollenkitt [168]. A simple and effective method was developed to reveal its presence or absence [169, 170]. The synthesis of pollenkitt is linked to tapetal degeneration [171] and plastids are implicated in its formation [104, 167]. In anemophilous plants, the plastids develop into elaioplasts which are resorbed by other tapetum cell components during degeneration. In entomophilous plants, the elaioplasts or tapetosomes (plastids accumulating lipids) are the more abundant organelles in the degenerating tapetum cytoplasm [167]. Tapetosomes are oil and flavonoid containing organelles in the tapetum that contribute to pollenkitt formation [172–174]. Pollenkitt is formed by the fusion of elaioplasts and spherosomes of tapetal cells during the late microspore stage [167]. After release in the locule, pollenkitt is deposited on the exine surface of the pollen grains, covering the exine ornamentalations at the onset of anther dehydration [171]. In the entomophilous Brassicaceae family, elaioplasts are involved in forming tryphine, which plays a role in adhesion of pollen to the stigma [104] (Table 1). A conditionally male sterile mutant that affects tryphine production in *Arabidopsis* is affected in pollen-stigma recognition [69]. Pollenkitt consists mainly of saturated and unsaturated lipids, carotenoids, flavonoids, low molecular weight proteins and carbohydrates [167, 175]. An additional role of pollenkitt in biotic pollination could be in preventing water loss and other damage [167].

6. Pollen metabolism and development: role of vacuoles and plastids

6.1. Role of vacuoles

Vacuoles appearing at several stages of pollen development are correlated with metabolic activity. Pollen mother cells, like undifferentiated meristematic cells, are originally devoid of vacuoles but at telophase II small roundish vacuoles start to develop. Vacuolisation can occur once or twice (depending on species) during further stages of development [18]. Cyclic vacuolisation is always followed by storage of starch in amyloplasts (Figure 3 B–D), which then leads to disappearance of vacuoles and formation of new cytoplasm. Vacuolisation plays a role in increasing the volume of the pollen grain with the formation of new cytoplasmic components such as mitochondria, amyloplasts, other cell components and cytoplasmic reserves. Vacuolisation therefore reflects metabolic activity in the developing microspores. Vacuolisation is also associated with the storage of pectins during intine cell wall synthesis [146]. In *Arabidopsis* a large vacuole is formed by fusion of smaller vacuoles; this large vacuole is converted to smaller vacuoles again after the first mitotic division [176]. Lytic vacuoles (lysosomes) are formed to degrade mitochondria, ribosomes and plastids [18]. Mature pollen has only small vesicles filled with carbohydrates, but in species producing pollinia rather than single pollen (e.g. massulate orchids) small vacuoles with watery content are present. Reduced vacuolisation at maturity may be required to reduce pollen size during presentation and dispersal [33]. Pollen vacuolisation is also affected by abiotic stresses such as drought and temperature stresses (Table 3). Heat stress was shown to reduce pollen release from anthers [177]. Vacuoles also store metabolites such as sugars and play a role in regulating sugar homeostasis, metabolic activity and growth processes [178]. Sucrose cleavage into hexoses by vacuolar invertases can regulate osmotic potential of cells [179] and this can be used as a defence mechanism against stresses such as drought (Table 3). Abiotic stresses in *Arabidopsis* induce vacuolar invertase, as well as a tonoplast-associated monosaccharide transporter (*ESL1*) in vascular parenchyma cells [180]. Regulation of cellular sugar fluxes between cytoplasm and vacuoles is important to regulate osmotic potential and pollen hydration and this could play a role under environmental stress conditions. Vacuolar invertases that are expressed in pollen grains have been identified [108, 109], but their role in regulating pollen metabolism under stress conditions requires further investigation.

6.2. Role of plastids

Plastids are commonly present as undifferentiated pro-plastids at the end of meiosis. They divide later to differentiate and accumulate starch [181, 182]. Plastid division occurs in the vegetative cell of pollen before starch engorgement. Usually, there are one or two waves of starch accumulation in amyloplasts during pollen grain development in gymnosperms and angiosperms [162, 181]. In some plant species, pro-plastids in the generative cells are degraded by lysosomes immediately after the first haploid mitosis [183]. Plastids also store fatty acids and alcohol intermediates for pollen wall synthesis, as evidenced by the male sterile mutant *defective pollen wall (dpw)* [184]. Starch stored in the amyloplasts of the vegetative cell is in most plants hydrolysed before anther opening and pollen dispersal (Figure 3C). Physico-chemical

properties of starch in plants with two cycles of starch synthesis vary between and within species [185]. Mature pollen can be starchy or starchless, depending on the presence or absence of starch grains in the vegetative cell amyloplasts (Table 1). This can be characteristic for plant families [185]. In some plants that flower throughout the year in the same environment pollen grains are always starchless (e.g., *Mercurialis annua*) [186]. Vice versa, in the case of *Parietaria judaica* which flowers from springtime to autumn, the proportion of starchy and starchless grains varies according to the season [187].

6.3. Adjustment of osmotic pressure and water balance in pollen

Like soluble sugars, starch stored in plastids can play a role in adjusting osmotic pressure, particularly during presentation and dispersal (Table 3). Stored carbohydrates in plastids or in the cytoplasm, soluble or insoluble, can be used to adjust turgor pressure and protect grains against desiccation [188]. Many genes are involved in starch biosynthesis throughout pollen development [189]. Drought and temperature stresses can severely affect starch accumulation, and absence of starch in mature pollen can be an indicator of pollen sterility [109, 110, 115, 190–192]. Endogenous starch is consumed during the first phases of pollen tube emission when pollen tube growth is at the expense of pollen reserves [193, 194]. After this autotrophic phase, pollen grains obtain carbohydrates and other substances from the stigma and style. Starch presence is not a direct indication of carbohydrate reserves present in pollen; hydrolysis of starch from amyloplasts increases soluble sugar levels in the cytoplasm and sugars are stored in the vacuole [188, 193]. Carbohydrates derived from starch hydrolysis in starch-less pollen grains alleviates the effect of heat and humidity stress during presentation and dispersal [193]. Starch in plants is normally phosphorylated. A tomato mutant lacking starch phosphorylation activity (*Legwd*) fails to degrade starch for pollen germination, resulting in sterile pollen [182]. Hydrolysis of starch supplies soluble osmotically active sugars which, together with amino acids such as proline, provide osmotic adjustment [195] (Tables 3 and 4). Regulation of turgor pressure is an essential aspect of pollen tube growth and elongation [196]. Osmo-regulation during the late maturation phase may function in the dehydration of pollen. Pollen dehydration is associated with the induction of proteins that play a role in drought response: dehydrins, aquaporins, heat shock and LEA proteins [197]. High levels of osmotin expression in mature tobacco pollen is another indicator of osmotic stress response [198]. Potassium ions [199, 200] and phospholipids can also regulate osmotic pressure and cell swelling in pollen [201]. Regulation of pollen osmotic potential and water content and the role carbohydrates play in this process are clearly important in pollen development. Abiotic stresses (cold, heat and drought) during meiosis affect sink strength of the tapetum [109, 110, 126, 191, 202], but the dynamics of carbohydrate metabolism at the gametophyte level remain poorly understood.

<i>Stage of development</i>	<i>Metabolic activity</i>	<i>Physiological effect</i>
Ripening*	<ul style="list-style-type: none"> Hydrolysis of starch Synthesis soluble carbohydrates, amino acids, peptides 	<ul style="list-style-type: none"> Molecules increase pollen turgor pressure

<i>Stage of development</i>	<i>Metabolic activity</i>	<i>Physiological effect</i>
Desiccation*	<ul style="list-style-type: none"> · Resorption of water by phloem of filament · Water redistributed to other flower parts · Evaporation through anther cuticle · Synthesis of protective molecules, proteins (LEA, dehydrins) 	<ul style="list-style-type: none"> · Desiccation leads to higher pollen osmotic pressure · Size of pollen grains affects desiccation
Presentation and dispersal	<ul style="list-style-type: none"> · Pollen water content is affected by humidity, temperature, content in osmotic molecules and their biosynthetic enzymes, levels of protective molecules 	<ul style="list-style-type: none"> · High relative humidity causes precocious rehydration and extra-stigmatic pollen tube emission (especially in recalcitrant pollen)
Rehydration and pollen tube emission	<ul style="list-style-type: none"> · Time for rehydration/pollen tube emission depends on water content, osmotic molecules, biosynthetic enzymes, stigma adhesion 	<ul style="list-style-type: none"> · The physiological state of the stigmatic surface plays an important role in pollen rehydration

(*): The ripening and desiccation stages can – at least in some species – be totally or partially overlapping.

Table 4. Synthesis of osmotically active components in pollen, and their effect at different stages of development. Orthodox and recalcitrant grains could have a similar physiological behaviour until the onset of desiccation, but the amount and quality of the osmotic molecules and the activity of their biosynthetic enzymes distinguish the two categories in the later stages of pollen development.

7. Consequences of synchrony and asynchrony in pollen development: Pollen competition

The synchrony of the first meiotic division is likely due to the presence of cytomictic channels that unite the cytoplasm of all the meiocytes present at meiosis within a loculus [203, 204]. These channels close during the meiotic inter-phase and synchrony can be lost from the second meiotic division onwards; the two nuclei within one meiocyte can divide independently, but a certain proportion (30–40% in *Lycopersicum peruvianum*) can still divide synchronously [205]. Nevertheless, the dissolution of the callose wall that keeps the tetrad cells together is synchronous and is controlled by callase, which is produced and released by the surrounding tapetal cells [95]. Meiotic asynchrony can cause the second haploid mitosis and other cellular processes (vacuolisation, starch hydrolysis storage in plastids, intine formation) to be asynchronous [206]. In orchids, the process of microspore development is synchronous because of the persistence of cytomictic channels throughout meiosis, uniting all the microspores of a loculus until pollen mitosis [33]. Pollen maturation is not a synchronous event from the first mitotic division onwards. Because a large amount of ovules needs pollinating in the ovary, the staggered pollen maturation in orchids may offer an advantage in that overcrowding and competition of germinating pollen on the stigma can be avoided [33].

At anthesis, the release of microspores is controlled by the sporophyte; all pollen grains from a loculus are dehydrated and released irrespective of their developmental stage. In addition, pollen desiccation at the end of pollen development affects all pollen grains of the anther at the same time. The mix of asynchronous and synchronic events during pollen development results in a mixture of pollen grains at slightly different stages of maturity; the difference in physiological stage means that different pollen grains may contain different amount of reserves when they are released together during anthesis. Asynchrony in pollen development is obvious from differences in starch engorgement, vacuolation and pollen size at different stages of development (Figure 3 B-D). Asynchrony can also explain why in vitro pollen germination tests show variable efficiency, particularly for some plant species and for plants grown under stressful circumstances. In vitro pollen germination issues may reflect the in vivo situation; the higher the asynchrony of microspore development, the higher is the percentage of unviable and immature pollen grains at maturity. Environmental stresses such as drought, frost, heat, high humidity (rain and mist) exacerbate the degree of developmental asynchrony [114, 207], causing a further reduction in viable pollen count. Application of heat stress is a common technique used for improving yield of haploid embryos during microspore embryogenesis [208, 209]. Through induction of asynchrony in pollen development, abiotic stresses can affect the production of viable pollen at the gametophytic level. Very little is known about this process and its molecular and physiological basis.

Asynchronous development is responsible for pollen competition. Competition between grains occurs at different stages: during development, after rehydration on the stigma and during pollen tube growth. Asynchronous development, combined with the fact that the haploid pollen grains have a different genetic composition due to recombination of the sporophyte genome during meiosis, leads to differences in ability to compete during pollen development and this presents a continuous selective force throughout male gametophyte development. The tapetum cells secrete nutritive substances synchronously, but the asynchronous pollen grains have a different capacity to use these substances for development, causing competition. Asynchrony in development and differences in genetic composition then lead to competition during rehydration and pollen tube growth and the speed of pollen rehydration depends on the orientation of the pore(s) with respect to the stigma surface. The competition to be the first to fertilise the ovule(s) is an important selective force in plant sexual reproduction and played an important role in both plant and animal evolution [210].

8. Duration and continuity of pollen development

Pollen development is normally a continuous process that is interrupted only by pollen presentation and dispersal. Pollen meiosis takes only a few hours, but the duration of pollen development after meiosis can vary widely and depends on the plant species. As a rule, annuals develop pollen faster than perennials and woody species: pollen development takes 8 days for the herbaceous perennial *Lycopersicum peruvianum* [211] and approximately 7 days for geophytic *Lilium* species [212], while 18 days are required for the grass *Phalaris tuberosa* [213]. However, in some plants the process can be interrupted at various stages before presentation and dispersal. In some woody plants from temperate environments, the process

can be paused once or twice at the microspore or bicellular stages. The ability of pollen development to be interrupted is an adaptation mechanism to protect pollen against extreme environmental conditions during summer or winter. Interruptions are more likely to occur in plants where pollen development takes longer, especially in temperate climates where unexpected harsh weather conditions can occur. Some gymnosperms (e.g. *Juniperus communis*) and woody perennial angiosperms (e.g. birch, elder and hazelnut) that disperse their pollen at the end of winter differentiate their flower buds in autumn when environmental conditions are favourable [214, 215]. Under severe winter conditions, flower development is arrested and resumed in early spring. In hazelnuts, this interruption occurs at the bi-cellular stage [215]. The developing pollen grains appear dormant and anther metabolism is repressed. The influx of substances from the mother plant and the activity of anther wall chloroplasts are also reduced, suggesting that developmental arrest may be regulated by the mother plant. In some species, developmental arrest occurs prior to pollen meiosis. In some Mediterranean plants, flower buds develop during late spring but stay dormant during the hot and dry summer and development resumes in autumn [216]. The dioecious bay laurel (*Laurus nobilis*) flower buds of both sexes develop in early autumn, they pause development in winter and flower ripening and pollination occurs during early springtime [163]. It is not known how this developmental arrest of pollen development is controlled at the molecular and physiological level, but it provides a powerful defence mechanism to protect pollen and maintain fertility under sub-optimal climatic conditions.

9. Pollen dehydration, presentation and anther dehiscence

9.1. Orthodox pollen and cross-pollination

In cross-pollinating plants, the flower opens at anthesis and the pollen is dispersed to reach other plants (chasmogamy). To survive dispersal in the environment, pollen needs to be in a dehydrated state with low metabolic activity (Figure 4) [217, 218]. This is the case for orthodox pollen which is dehydration-tolerant and is dispersed with low water content (<20%). Orthodox pollen can travel over larger distances without losing viability [160, 219]. Near anthesis, rapid extension of the anther filament seals the xylem, interrupting sap flow to the anther. The phloem redistributes the locular content to other plant parts [160, 220, 221]. The epidermis and endothecium layers of the anther wall dehydrate and pollen grain hydration levels reach an equilibrium with the environment [222]. Environmental parameters such as temperature and relative air humidity influence pollen water content [186] and osmotic adjustment is used to balance water content in function of environmental conditions (Table 4) [27]. Orthodox pollen also has low metabolic homeostasis to prevent cellular damage during dispersal [160]. The duration of developmental arrest and viability of pollen depends on environmental conditions at dispersal and the type of reserve substances present in the pollen (Table 4) [27, 188]. These defence mechanisms protecting pollen grains during presentation, dispersal and pollination vary depending on the degree and duration of dehydration during dispersal and depend on whether plants are anemo- or zoophilous pollinators (Table 5) [27, 160, 223]. Relative air humidity can adversely affect pollination efficiency because absorption of water from the

environment can lead to precocious pollen tube emission when the correct hydrated state is reached (Figure 3E) [160]. Entomophilous pollen is also affected by compounds that are secreted by the insect carrier (e.g. bees) [224–226]. Plants producing orthodox pollen are potentially out-crossing; both out-crossing and self-pollination can occur in these plant species, unless there is a self-incompatibility system in place to prevent self-pollination [227].

Type of defence	Defence mechanism	Stage affected	
		Presentation	Dispersal
Structural, species-specific	<ul style="list-style-type: none"> · Close proximity of small herbaceous (social) plants · Grains protected inside anther until dispersal: <ul style="list-style-type: none"> ◦ Pollinia of massulate orchids ◦ Gradual dispersal, e.g. poricidal anthers of Ericaceae, Solanaceae · Anthers exposing and protecting pollen inside the corolla 	X	X
Ecological	<ul style="list-style-type: none"> · Pollen is presented during short periods with more favourable conditions ◦ Night pollination in dry habitats, e.g. Cactaceae ◦ During dry and sunny periods of the day, e.g. Gymnosperms 	X	X
Cytological	<ul style="list-style-type: none"> · Synthesis of molecules that protect pollen under stress conditions: carbohydrates, proteins and enzymes · Intine is thick and stores water, regulating the water content of the cytoplasm 	X	X

Table 5. Common types of modalities present in different angiosperms in order to reduce and/or avoid the harmful effects of the environment during pollen presentation and dispersal.

9.2. Recalcitrant pollen and self-pollination

In self-pollinating plants, pollen does not have to travel far to pollinate and therefore does not need to undergo severe dehydration at maturity. These plants produce recalcitrant pollen grains which are dispersed with high relative water content (30–70%); pollen remains metabolically active at dispersal and continues to develop to the point of germination (reduced developmental arrest). Recalcitrant pollen grains are dehydration-sensitive and are typically very short-lived and highly sensitive to variation in relative air humidity [160] (Figure 3 A1 and A2; Figure 4). However, cross-pollination with recalcitrant pollen is possible but is restricted to proximate flowers only [228]. Some plant species produce both chasmogamic and cleistogamic flowers, thereby increasing the chance of reproductive success [227]. In crop

species (e.g. wheat, barley, rice), cleistogamic breeding systems may have been selected during domestication to limit gene flow and preserve preferred gene combinations [229–233]. The absence of pollen presentation in cleistogamic self-pollinating plants is thought to be a protection against abiotic stresses such as drought and heat, as pollen number is considered less of a constraint for pollination in cleistogamic compared to chasmogamic species [227, 234]. Some crop species still have both cleistogamic and chasmogamic varieties [232, 235, 236]. Cleistogamic rice varieties were shown to be more tolerant to heat stress at flowering compared to non-cleistogamic lines [237]. However, recalcitrant pollen (e.g. maize) can lose water quickly, especially at low air humidity [238] and many cleistogamic crop species (e.g. cereals, legumes, Solanaceae) have well-documented pollen sterility problems. These problems occur when plants experience stress at the young microspore stage or anthesis [25, 115, 191, 239–241]. Sterility in these cases may be inflicted earlier in development and may not be due to interference with pollen presentation and dispersal [242, 243]. This may indicate that cleistogamy *per se* may help avoiding pollen dispersal, but it may not offer protection against abiotic stresses that occur at other periods of flowering. Genetic manipulations and hybrid breeding in crop species have sparked renewed interest in controlling the breeding system of some crop species [231, 244–246]. Some progress has been made in recent years to identify the genes associated with the cleistogamy trait and flower opening in rice, wheat and barley [246–249]. This research will lead to a better understanding of the genetic basis of cleistogamy and chasmogamy and the implications for abiotic stress tolerance in crop plants.

9.3. Pollen size, shape and anther dehiscence

The size of mature pollen grains at dispersal varies from less than 15 to 200 µm in diameter, with an average size of 70–100 µm in the desiccated state. The variation in pollen size has been related to the stigma size [250] and does not always correlate with water content (Table 1) [160]. Pollen grain volume increases progressively from the young microspore stage to maturity but is generally restricted by available locular space and the type of pollen dispersal unit in different species [168, 251]. The dehydration process in orthodox pollen leads to a change in shape and size of pollen grains and the harmomegathic properties of the cell wall play an important role in this process (Figure 4; Table 6) [154]. Recalcitrant pollen do not have furrows to facilitate mechanical folding of the cell wall in response to dehydration and pollen remain spherical (Figure 4; Table 6).

Pollen release from the anther requires thickening of the secondary wall of the endothelial layer (= mechanical layer) and dehydration of the epidermis [163, 252–254] (Figure 1). Dehiscence mutants in *Arabidopsis* affect secondary wall thickening and cause male sterility; these mutants were shown to affect transcription factor genes *MYB26*, *NST1* and *NST2* [255–257]. Secondary cell wall thickening can also control temporary re-closure of the anther during rainy or misty weather [258, 259]. Dehydration of the epidermis is associated with increased abscisic acid (ABA) levels [260] and induction of dehydrin-like proteins [261]. Aquaporins regulate the movement of water during anther opening [262, 263]. Cells of the inter-locular septum are ruptured as a result of PCD, causing the joining of both locules of one theca – see Figures in Keijzer CJ [171] and Bonner LJ and Dickinson HG [264]. The locule volume increases and

absorption of the locular fluid is accelerated [220, 265–268]. The locular content is re-distributed to other plant parts via the elongating anther filament [160, 221] and aquaporins may facilitate the movement of water through the anther wall membranes [262]. A cell death response in the stomium then causes the anther to open and pollen grains dehisce with the help of tension caused by secondary wall thickening [253]. Depending on the plant species, the stomium can rupture completely (from the top of the anther to the base), partially, or form pores for pollen dispersal [266, 267, 269]. Plant hormones regulating senescence and cell death such as auxin, jasmonic acid and ethylene play a role in anther opening and pollen dehiscence [252, 270–273]. The elongation of the anther filament in some plant species is required to expose the anthers from the flower to facilitate dispersal (Table 5) [274].

Pollination in plants requires favourable interactions between pollen morphological factors and environmental conditions (Tables 6 and 7) [275]. The size and shape of pollen grains, together with the events in the anther wall regulating dehiscence all collaborate to determine desiccation time, pollen viability and pollination success (Tables 6 and 7). Variation in relative air humidity, together with abiotic stresses that affect relative humidity (heat, drought, cold stress), cause problems with pollen presentation, anther opening, dehiscence [276, 277] and pollen tube growth [32]. Precocious germination while still in the anther [278, 279], or while waiting for a pollinator to disperse the pollen (Figure 3E) [280, 281], is due to inappropriate levels of humidity. Plants have evolved clever species-specific adaptation mechanisms such as dehiscence at particular times of the day [282], dispersal as single pollen or aggregates [168, 283], active dispersal by explosive forces rupturing the anther (e.g. *Ricinus communis*) and interaction with grooming insects [284, 285].

	<i>Pollen stages</i>	<i>Processes affected by abiotic stress</i>
Pollen development	· Meiosis	<ul style="list-style-type: none"> Drought prevents secretion of the locular fluid Drought during pollen development influences volume increase of the different floral parts
	· Tetrad stage	<ul style="list-style-type: none"> High/low temperatures and drought lead to consumption of starch reserves and carbohydrate starvation in anthers, affecting sugar delivery to pollen
	· Microspore stage	<ul style="list-style-type: none"> High/low temperatures and drought lead to consumption of starch reserves and carbohydrate starvation in anthers, affecting sugar delivery to pollen
	<ul style="list-style-type: none"> First haploid mitosis (asynchronous) Bi-cellular/tri-cellular stage 	<ul style="list-style-type: none"> Drought during anther and pollen desiccation prevents transport of locular fluid water to other floral parts
Anther and pollen desiccation		<ul style="list-style-type: none"> High air relative humidity prevents anther and pollen desiccation Too low relative humidity of the air accelerates anther and pollen desiccation
Pollen presentation ^(*)		<ul style="list-style-type: none"> Too low air relative humidity affects pollen viability, especially in recalcitrant species

Pollen stages	Processes affected by abiotic stress
	<ul style="list-style-type: none"> • High air relative humidity induces precocious rehydration of pollen grains and pollen tube emission • Low or high temperature extends or reduces pollen presentation • Drought reduces flower longevity
Pollen dispersal	<ul style="list-style-type: none"> • Low air relative humidity affects pollen viability • High air relative humidity induces precocious pollen rehydration and can prevent anther dehiscence • Some volatile compounds emitted by bees affect pollen viability
Pollen rehydration	<ul style="list-style-type: none"> • Low air relative humidity prevents pollen rehydration and affects water availability from the stigma

(*): This phase is absent when pollen leaves the anther when it opens (e.g. Poaceae) or is launched from the anther (e.g. castor bean)

Table 7. Stages of male gametophyte development in angiosperms and processes affected by abiotic stresses.

9.4. Breeding systems and pollen:ovule ratio

The pollen:ovule ratio (P/O) has traditionally been used as a rough estimator of plant breeding systems (Cruden 2000), but little is known about the effect of environmental stresses on this ratio. When pollen is dispersed in aggregates of hundreds of grains (e.g. massulate orchids), the locular space is restricted and limited locular fluid limits nutrition and volume increase [34]. Changes in pollen volume can be measured under optimal or stressed conditions [28, 286]. Pollen dispersed as aggregates provides greater pollination success when the ovary contains multiple ovules [168, 287] and water loss during presentation and dispersal under heat and drought conditions affects only the externally exposed pollen grains and not the internal ones. To improve pollination success, some plants produce different types of pollen (different size, shape, colour, carbohydrate and water content) in one flower. One type, fecundative pollen, is fertile and able to emit the pollen tube and fertilise, while the other type is sterile nutritive pollen that serves as a reward for pollinators who – at the same time – get dusted with fecundative pollen [288]. The flower morphology can affect accessibility of pollen by different pollinators. Self-incompatible dimorphic *Primula* species have two different flower types with reciprocal anther and style length, producing pollen with different water content depending on the position and exposure of the anthers with respect to the corolla tube [289, 290]. Three flower types, producing three types of pollen grains, occur in trimorphic species (e.g. *Lythrum salicaria*) [289, 291]. The differences in flower morphology result in non-random mating patterns in plant populations and may play an important role in pollinator selection and adaptation to different environments [292, 293].

10. Conclusions

The diversity in adaptation mechanisms available in nature to secure reproductive success in angiosperms is considerable (Tables 6 and 7). This diversity can serve as a valuable resource to advance our insights into stress adaptation mechanisms that will benefit breeding strategies for crop species. Cytological and morphological studies, combined with other science disciplines (physiology, genetics and genomics) will continue to improve our understanding of pollen development and its adaptation to the environment. The number of genes and mutants involved in male reproduction is steadily increasing [294], but several research areas require further attention:

- Two crucial stages of anther development are strongly affected by environmental conditions. Until dehiscence, anthers are protected by the calix and corolla, but for pollen dispersal, anthers need to be exposed. Both flower opening and anther dehiscence are strongly influenced by the environment [171, 184]. Secondly, the secretion functions of the anther tapetum are strongly affected by abiotic stresses. Tapetum cells are highly specialised secretion cells that loose their inner cell walls, effectively turning them into natural protoplasts and making them very vulnerable to water stress [82]. Drought stress at meiosis reduces locular fluid secretion [115], causing malnutrition and asynchrony of the developing pollen grains. Interestingly, some plant species are adapted to growth in very arid environments and expose pollen during the hot season, yet always have a very reduced volume of locular fluid (e.g. *Eucalyptus* and *Acacia* species in Australia). *Eucalyptus rhodantha* can resist temperatures higher than 50°C for several days without significant reduction in pollen viability [295]. It is important to understand how the tapetum of these plants manages to provide sufficient nutrients to sustain pollen development. The available locular space and the capacity to store locular fluid are abundant in plants dispersing solitary pollen, but very reduced when grains are dispersed as polyads (e.g. pollinia) [82]. Abundant locular fluid is considered a ‘primitive’ character in land plants and is a characteristic shared by all gymnosperms [160]. During evolution, locular volume has been gradually reduced and/or replaced by polyad dispersal, possibly as an adaptation to drier environments or to allow pollen presentation over longer periods of time (e.g. massulate orchids) [33, 105]. Orchid species can have monad or pollinia dispersal units [296]; the more primitive species have monad and tetrad pollen with abundant locular fluid, while the more evolved species disperse pollinia and produce very little locular fluid [33]. It remains to be established whether/how reduced locular fluid volume and compound pollen dispersed over longer periods of time could benefit sexual reproduction in arid environments and orchid species could be used for this research. Various other adaptation mechanisms could alleviate the effect of abiotic stresses, including shorter duration of pollen development, night – rather than day – pollination, deposition of a thicker protective intine wall, dispersal of compound rather than single pollen can all reduce the negative effect of stresses [160, 279].
- The control of pollen number, size and shape is another poorly understood aspect of pollen development. Pollen development is started (meiosis) and terminated (anther dehiscence) at a fixed moment. When environmental conditions induce various degrees of asynchrony

throughout pollen development, this leads to decreased numbers of viable pollen at anthesis. Larger pollen numbers could be obtained in plants with larger anthers. Anther size is a trait that has been used for selection of cold tolerance in rice [297] and the growth hormone gibberellic acid plays an important role in controlling stamen development [298]. Elucidating the mechanism of interrupting or pausing pollen development under unfavourable conditions may also provide useful information about avoiding stress damage. Understanding these mechanisms will require a better understanding of the signals driving gametophyte development *per se*. The haploid genome of the male gametophyte is derived from the sporophyte, but very little is known about its functionality in regulating pollen-specific development and metabolism. Achieving this challenge is now within reach, thanks to sensitive new-generation transcriptome analysis techniques [29, 71, 299].

- It is important to understand the signalling mechanisms between mother plant and male gametophyte. Some crucial steps in pollen development (meiosis, tapetal activity and anther dehiscence) are clearly under sporophytic control. The high sensitivity to abiotic stresses of the meiotic, young microspore and anthesis stages indicates that sporophytic signals are critical in controlling male gametophyte development. Stress-proofing crop plants may therefore have to start by understanding the sporophyte signals (sink-source relationships, carbohydrate and hormone signalling, control of PCD during tapetum degeneration and anther dehiscence). It has been known for some time that treatments with one stress or with the stress hormone abscisic acid (ABA) can improve tolerance to another stress – a process called stress ‘hardening’ or ‘priming’ [300–305]. More recent studies in rice have shown that stress treatments at the vegetative stage can affect abiotic stress tolerance during flowering and reactive oxygen species (ROS) signalling could play a role in this sporophytic signalling event [306]. But evidence for involvement of genomic imprinting and epigenetic mechanisms in sporophyte-gametophyte signalling is also mounting [307–309].
- The importance of air relative humidity in pollen development has so far been grossly underestimated. The growing area of staple crops such as cereals is increasingly extending into environments that require different adaptations of pollen development. For instance, tropical rice is grown in temperate climate zones and temperate climate wheat is grown in humid tropical environments [310–312]. Air humidity and climatic conditions modifying atmospheric humidity (rain, fog, cold, heat and drought) have a dramatic effect on plant species producing orthodox and recalcitrant pollen, causing asynchrony and reducing pollen number and fertility. The dynamics of water relations and osmotic regulation in pollen grains and their interactions with the environment are research topics that need urgent attention. Adapting the breeding system of crop species (self- versus cross-pollination) may offer opportunities for improved protection of pollen during dispersal, but the trade-offs between chasmogamy and cleistogamy in terms of abiotic stress tolerance require more detailed investigations.

11. Appendix

Term	Definition
• Meiocyte, pollen mother cell	Sporophytic cell in the centre of the anther that is destined to undergo meiosis and generate haploid pollen grains.
• Microspore	Alternative term used to refer to a pollen grain, but mainly used for the earlier uni-nucleate stages of pollen development. Young microspores refer to the first stage of pollen development, i.e. the cells released from the tetrad after meiosis. Microspores develop into the male gametophyte.
• Tapetum	Inner layer of the anther wall surrounding the meiocytes and loculus of the anther. Consists of secretory apoptotic cells that nourish and regulate pollen development. The tapetum degenerates, producing pollenkitt and other substances that cause pollen grains to aggregate.
• Cleistogamy/chasmogamy	Cleistogamy refers to automatic self-pollinating plants that do not open their flowers before pollen dispersal. In contrast, chasmogamy refers to plants that do open their flowers to release pollen in the environment for dispersal by animals or wind (potential cross-pollinators).
• Pollen Dispersal Unit	Pollen grains can be dispersed as single grains (monads) or as aggregates of several pollen grains kept together by viscous fluids or filaments (polyads). Tetrads derived from a single meiocyte can stay together in groups of four, united by common walls. In orchids, many packed tetrads can be arranged in different ways to form pollinia containing hundreds or thousands of pollen grains.
• Monads, polyads, pollinia	See pollen dispersal unit.
• Orthodox/recalcitrant pollen	Based on water content at dispersal, pollen grains can be classified as orthodox or recalcitrant. Orthodox pollen is desiccation-resistant and has a low water content (2–20%). Recalcitrant pollen is desiccation-sensitive, with water content between 20% and 50%. Orthodox and recalcitrant pollen grains both have advantages and disadvantages at pollination.
• Male germ unit	Is the association of a vegetative nucleus with a generative cell or two sperm cells to form a functional male reproductive unit in angiosperms. The term ‘unit’ reflects the close connection between the sperm cells and the vegetative nucleus.
• Septate/aseptate anthers	In septate anthers, in contrast to aseptate anthers, the meiocytes are separated by a wall (septum), dividing the locule in smaller compartments filled with pollen grains.
• Pollen presentation	Is the process of pollen exposure for dispersal to reach the stigma for pollination. Pollen presentation involves interaction between the anther and other floral parts. Primary presentation occurs when pollen grains are

Term	Definition
	exposed in the anther. Secondary presentation involves developmental relocation of pollen from the anther to another floral organ. Pollen grains are not presented by the anther when they are launched using different mechanisms.
• Zoophilous, entomophilous and anemophilous pollen	Pollen dispersal by animals, insects and wind, respectively.
• Pollen engorgement	Pollen maturation is associated with accumulation of starch granules in the cytoplasm. This process is called engorgement.
• Harmomegathy	The capacity of pollen grains to change shape in response to a decrease in volume during dehydration and prior to the development arrest state. This dynamic process is controlled by the mechanical properties of the cell wall (furrows) and can be reversed by rehydration on the stigma. When pores are absent, this increase and decrease in volume is due to the elasticity of exine and intine.
• Furrow	A fold region where the exine cell wall has reduced thickness, whilst intine is thicker. Furrows allow the cell wall to collapse to comply with the decrease in pollen volume during dehydration and increase volume during rehydration.
• Development arrest state	Term used to indicate the state of physiological and metabolic arrest when pollen grains reduce water content before dispersal.
• Of the locular fluid changes	Central cavity in the anther where pollen grains develop. The loculus is filled with the locular fluid which is secreted by the tapetum and serves to nurture pollen. In cross-section, anthers show four locules. The composition of the locule fluid changes during pollen development, and before anther dehiscence the fluid is reabsorbed by the filament or other floral parts to allow pollen presentation. The locular fluid is abundant in anthers with monad and tetrad pollen, but is reduced in species with pollinia or where grains are tightly packed.
• Mechanical layer	External cell layer of the anther wall where, after tapetum degeneration, cells develop lignified wall thickenings. The mechanical layer is responsible for anther opening and pollen exposure
• Pollenkitt	Hydrophobic glue derived from the degeneration of the tapetum, composed of saturated and unsaturated lipids, carotenoids, flavonoids, proteins and carbohydrates. Pollenkitt makes grains stick to the anther, to the pollinator body and to the stigma surface.
• Pollen viability	Term used to indicate the percentage of viable pollen (i.e., able to emit pollen tubes and fertilise). Pollen viability can be assessed by hand pollination, in vitro germination and several methods evaluating physico-

Term	Definition
	chemical parameters of pollen (e.g., plasma membrane intactness, the presence/abundance of some molecules or enzymes).
• Sporopollenin	Chemically and biologically resistant and elastic substance forming the building block of the exine cell wall. Sporopollenin consists of a mixture of carotene and carotenoid esters.
• Exine	External discontinuous cell wall of pollen grains. Exine is elastic, is composed of sporopollenin and has an opening called the pollen germination pore or aperture.
• Intine	Inner continuous pecto-cellulosic wall of pollen grains. The intine structure is more complex at the apertures and furrows where pollen tubes will be emitted. The intine wall becomes continuous with the pecto-cellulosic wall of the pollen tube during germination.
• Callose	Polymer of glucose residues linked together through β -1,3-linkages. Callose is deposited during meiosis to separate the meiocytes and tetrad cells during meiosis. Callose represents a molecular filter to separate cells and is degraded by callase separated by the tapetum (β -1,3-glucanase).
• Pollen desiccation and water content	Pollen grains desiccate before dispersal to reach equilibrium with environmental conditions. Metabolism is slowed down to better resist the negative effects of the environment (high or low temperature and relative humidity). Orthodox and recalcitrant pollen have different water contents at dispersal.
• Secreted by the gymnosperm ovule	Liquid secreted by the ovule and exposed outside the stigma. When pollen grains land in the pollination drop, they rehydrate and germinate.
• Pollination syndrome	Term to describe the pollination traits that plants use in their natural environment to move from one flower to another, using different vectors. Plant can use abiotic (wind, water), as well as biotic (bees, birds) vectors to transfer pollen grains.
• Pollen competition	Haploid pollen grains differ in their genomic composition (recombination during meiosis) and therefore behave differently during development, pollen tube germination and in response to environmental challenges. This leads to competition between pollen grains. Pollen competition is an example of rapid Darwinian selection.

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Transcriptional Network Involved in Drought Response and Adaptation in Cereals

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Abstract

Drought is the major abiotic stress in many wheat environments, decreasing grain yields and farmer's income. Finding ways to improve drought tolerance in wheat is therefore a global effort. Transcription factors (TFs) play important roles in drought tolerance by stimulating plant's protective genome activities in response to heat and water limitation. TFs are specialized proteins which can bind to specific DNA elements in gene promoters and modulate gene expression in response to various external and internal stimuli. Thus TFs is a crucial part of plant signal transduction pathway mediated by signal receptors, phytohormones and other regulatory compounds. The activities of TFs are closely related to their structure, and their binding specificity is determined by the homo-/hetero-dimerization of TFs. The expression of downstream genes may produce a subset of TFs or regulate other functional proteins involved in physiological drought adaptation. Thus, the hierarchic regulations of TF activities, downstream gene expression and protein–protein interaction comprise a complex regulatory network, which participates in drought response and adaptation in cereal crops. Basic mechanisms of this regulatory network have been described, but more insight is needed to find new tools for enhancing cereals' adaptation to drought stress.

Keywords: Abiotic stress, cereals, drought, regulatory networks, transcription factors

1. Introduction

Drought is the major environmental factor that limits crop growth and yield globally. Improving crop performance under water limiting conditions is, therefore, an important research focus of plant scientists around the world. Limited water availability evokes adaptive physiological responses regulated by changes in expression of numerous stress-responsive genes.

Transcription factors (TFs) are groups of proteins that bind to specific regulatory DNA elements located in gene promoters upstream of transcription initiation sites, repressing or activating target gene expression. Intensive research in recent years has shown that temporal and spatial modulation of stress-related TFs provides an efficient way for plants to deal with unfavourable growth conditions. TFs involved in drought response were identified and characterized in various cereal species, including all major food crops such as in rice [1–4], wheat [5–8], barley [9, 10], and maize [11, 12]. Overexpression of several target genes encoding stress-responsive TFs led to improved survival rate under water limitation in transgenic plants [4, 10, 12–14].

The activities of TFs are closely related to their structure. TFs usually contain a DNA-binding domain (DBD) and a transcriptional activation domain (TAD) [15]. The DBD enables TFs to bind with specific promoter elements of target genes, and TAD mediates regulation of the downstream gene either directly or in cooperation with other proteins. TAD usually represents a low-complexity sequence that prevents protein self-folding and facilitates protein–protein interactions (PPI). The transcriptional response to drought in cereals is controlled by a large number of TFs, which have been grouped into several different families based on their structure and binding specificity. The main TFs discussed in this article belong to the following families: the DRE-binding protein/C-repeat binding factors (DREB/CBF) [1, 13, 16, 17], the NAM/ATAF1/CUC2 (NAC) factors [18, 19], the MYB family [20, 21], the WRKY family [9, 22], the basic leucine zipper family (bZIP) [14, 23–25], and the homeodomain-leucine-zipper (HD-Zip) family [26–28].

When plants suffer water deficiency, receptors from the cell membrane/cell wall sense the extracellular stress signals and convert them into intracellular secondary messengers such as Ca^{2+} and inositol phosphate [29]. How exactly the signal is transmitted toward gene activation is still poorly understood and is a subject of intensive multidisciplinary investigations. However, it is well agreed that plant hormones, especially abscisic acid (ABA), play significant role in drought stress-related transcription, in many cases through modulating phosphorylation status of transcription factors and other regulatory proteins.

The objective of this article is to review the involvement of TFs in drought response and adaptation in cereals and to illuminate the complexity of the factors and processes involved. The article is subdivided into four sections, which will (1) give examples of drought-related hierarchy in TF interactions regulated by plant hormones, (2) provide an overview of major families of cereal TFs involved in drought response, (3) overview existing data on TF target gene networks activated in response to drought, and (4) describe the homo- and heterodimerization in relation to TF's activities.

2. Plant hormone crosstalk in drought relevant regulatory pathways

Phytohormones play critical roles in linking the stress-responsive signaling cascades. ABA is a key plant hormone that functions as a link between environmental stress reception and adaptive transcriptional programs such as the regulation of cellular mechanisms, carbohydrate

and lipid metabolism. Similar to ABA, gibberellic acid (GA) and jasmonic acid (JA) play important functions in cellular stress network signaling. Different receptors have been reported to recognize and bind with these plant growth regulators in order to activate or modulate downstream responses [30].

Drought and ABA-mediated signals are perceived through three main pathways by different receptors (see Figure 1). The first receptor PYR/PYL/RCARs (PYRABACTIN RESISTANCE / PYRABACTIN RESISTANCE-LIKE / REGULATORY COMPONENT OF ABA RECEPTOR-SPYR) binds ABA and inactivates the type 2C protein phosphatases (PP2Cs), which leads to the accumulation of SNF1-RELATED PROTEIN KINASES (SnRK2s) [30]. SnRK2s activate ABA-responsive TFs such as *Arabidopsis* AREB1, AREB2, and AREB3. These TFs regulate ABA-dependent gene expression involved in several physiological processes such as the movement of stomatal guard cells, thereby increasing the tolerance to drought [30]. AtMYB44 is one of the TFs that negatively regulate the target genes coding PP2Cs, which leads to stomatal closure and reduced transpiration losses [31]. Protein-coupled receptor-type G proteins (GTGs) such as GTG1/GTG2 are involved in the second ABA reception pathway, which was first reported in *Arabidopsis* [30]. GTG1/GTG2 proteins are membrane-localized receptors with functions in seedling and pollen tube growth and development, acting through voltage-dependent anion channels. The third ABA receptor is the H subunit of Mg-chelatase (CHLH/ABAR), which regulates the lipid metabolism linked to drought tolerance in plants [30].

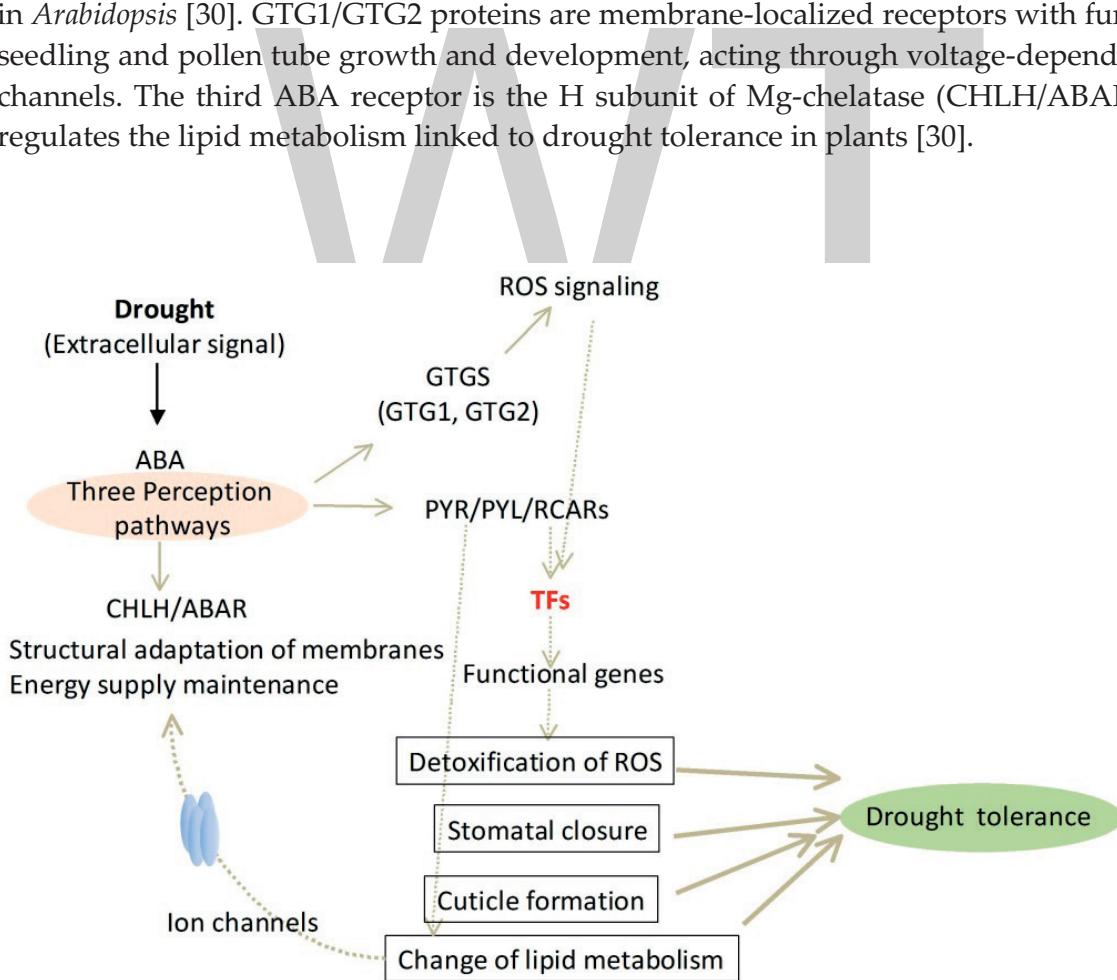


Figure 1. A schematic model of the signal chain from drought stress perception to physiological responses and drought tolerance.

It was also demonstrated that ABA can increase the transcription level of reactive oxygen species (ROS) network genes [30]. ROS are reactive oxygen-based molecules such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which not only toxically damage cells through membrane peroxidation and de-esterification under environmental stresses but also trigger stress endurance in plants [30]. For example, ABA has been shown to trigger the activity of cytosolic aldehyde oxidase and xanthine dehydrogenase, which separately produce H_2O_2 and O_2^- in drought [32].

The GA receptor GA INSENSITIVE DWARF1 (GID1) was reported from rice and is a homolog of the *Arabidopsis* GID1a/b/c [30]. GA-responsive TFs GRAS (GA insensitive [GAI], REPRESSOR of *ga1-3* [RGA], and SCARECROW [SCR]) are GA signaling repressors involved in GA-controlled plant development [30]. Subgroup of GRAS, called DELLA proteins, can interact with GID1 and lead to DELLA protein degradation. The downstream gene of DELLA TFs encoding a RING-H2 zinc finger factor XERICO is involved in ABA and GA transduction pathways under abiotic stresses [30]. Further, the DELLA protein RGL3 can be responsive to JA and interact with the JA regulator OsJAZ (jasmonic acid ZIM-domain protein) under drought [30]. Thus, DELLA proteins can be considered as the interface of ABA, GA, and JA signaling pathways in response to water deficiency [30].

The regulation network of TFs plays an important role in stress-relevant hierarchic regulatory pathways. OsNAC10, a NAC TF, can up-regulate the downstream genes encoding AP2 and WRKY TFs involved in ROS detoxification and scavenging for drought response through the ABA synthesis pathway. The mechanisms of plant response to drought include cell wall development and cuticle formation [30]. The promoter region of the gene OsNAC6 contains various recognition sites such as ABREs, MYBRS, MYCRS, W-boxes, and GCC boxes, which can be separately recognized by TFs AREB/ABF, MYB, MYC, WRKY, and ERF [33]. These TFs are likely to bind to the corresponding *cis*-elements and co-regulate the expression of OsNAC6 that participate in the ABA induction pathway and abiotic stress response in plants. In the bZIP family, the gene encoding OsbZIP12 was also found to have MYBRS, MYCRS, and W-box motifs in its promoter region, which can be recognized by TFs MYB, MYC, and WRKY, respectively [34]. Besides, OsNAC5 and OsbZip23 might co-regulate the expression of the downstream gene OsLEA3 since both of them enhance the transcription level of OsLEA3 [35]. OsDREB1F might interact directly/indirectly with some bZIP family members in the ABA-dependent pathway that activate transcription of the ABA responsive genes *rd29B* and *RAB18* [3]. However, more in-depth studies are needed to identify these events and to explain the underlying mechanism.

3. Major families of cereal TFs involved in drought response

Transcription factors are classified into several family groups mainly based on characteristic amino acid sequences of its conserved DBDs [36, 37]. Of these, the families DREB/CBF, NAC, MYB, WRKY, bZIP, and HD-Zip are the main TFs involved in drought. Their structural features, classification, and representative family members in cereals are summarized in Table 1 and Figure 2.

TF	Species	TFs name	Cis-element recognition	Downstream genes	Accession/or locus number	Reference	ABA (D/I)	Transgenic Plants	Stress inducible/tolerance
Rice		OsDREB1A	DRE/CRT (G/ACCCGAC)	e.g., <i>cov15A, rd29A, rd17</i>	AP001168	[1, 129]	D	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		OsDREB1E	DRE/CRT (G/ACCCGAC)	U	AY785896	[44]	I	Rice	Drought
		OsDREB1F	DRE/CRT (G/ACCCGAC)	<i>rd29A, COR15a, rd29B, RAB18</i>	AY345234	[3]	D/I	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		OsDREB1G	DRE/CRT (G/ACCCGAC)	U	XM_483622	[44]	I	Rice	Drought
		OsDREB2A	DRE/CRT (G/ACCCGAC)	U	AF005971	[45]	I	Rice	Drought
		OsDREB2B	DRE/CRT (G/ACCCGAC)	U	AK099221	[44]	I	Rice	Drought
DREB	Wheat	TaDREB1	DRE/CRT (TACCGACAT)	<i>rd29A</i>	AF03376	[41]	I	Rice, <i>Arabidopsis</i>	Drought, salt, cold
		TaDREB2	DRE/CRT (G/ACCCGAC)	e.g., <i>TaRAB16.5, TaNtZ2, TaWt10</i>	GU785008	[13]	I	Barley, wheat	Frost, drought
		TaDREB3	DRE/CRT (G/ATCCGAC)	e.g., <i>TaRAB16.5, TaNtZ2, TaWt10</i>	GU785009	[13]	I	Barley, wheat	Frost, drought
Barley		HvDREB1	DRE/CRT (G/ACCCGAC)	<i>rd29A</i>	DQ012941	[47]	D/I	<i>Arabidopsis</i>	Drought, salt, cold
		HvCBP4	DRE/CRT (G/ATCCGAC)	e.g., <i>Atise, LRR, C4P450</i>	AF298230	[48]	I	Rice	Drought, salt, cold
Maize		ZmDREB1A	DRE/CRT (G/ACCCGAC)	e.g., <i>COR15A, KIN1, KIN2</i>	AF50481	[11]	I	<i>Arabidopsis</i>	Drought, cold, salt
		ZmDREB2A	DRE/CRT (G/ACCCGAC)	<i>rd29A, rd29B</i>	AY108198	[110]	I	<i>Arabidopsis</i>	Drought, heat
		ZmDREB2.7	DRE/CRT (G/ACCCGAC)	U	GRMZMP2028386	[46]	I	<i>Arabidopsis</i>	Drought
		OsNAC5	NACRS (CACG)	<i>OslEA3, Os06g0631200</i>	AK104275	[35, 130]	D	Rice	Drought, salt, cold
		OsNAC6	NACRS (CACG)	<i>AK104277, AK110725</i>	B028185.1	[33, 35, 58]	D	Rice	Drought, cold, salt
Rice		OsNAC9	NACRS	e.g., NCED, Ca ²⁺ -ATPase, CCR	GSE31855	[59]	U	Rice	Drought
		OsNAC10	U	U	U	[60]	D	Rice	Drought, salinity
		OsNAC52	NACRS	e.g., <i>rd29A, rd29B, RD22</i>	AAT44250	[61]	D	<i>Arabidopsis</i>	Drought
		ONAC045	NACRS	<i>OsLEA3-1, OsPMI</i>	CTB29509	[19]	D	Rice	Drought, salt
		SNAC1	U	U	AK067690	[18]	D	Rice	Drought, salt
NAC		TaNAC-2	NACRS	e.g., <i>rd29A, rd29B, RD22</i>	U	[52]	D/I	<i>Arabidopsis</i>	Drought
		TaNAC67	NACRS	<i>DRE2A, COR15, AB12</i>	KF646593	[54]	D	<i>Arabidopsis</i>	Drought, salt, cold
		TaNAC69-1	U	<i>Chitinase, ZIM, glycosidase I</i>	U	[53]	U	Wheat	Drought
		ZmSNAC1	U	U	U	[131]	U	Wheat	Drought, salinity, heat
		ZmSNAC052	U	U	KM987612	[55]	U	N	
Maize		Zma000584	U	U	KP283536	[55]	U	N	
		Zma006493	U	U	KM670443	[55]	U	N	
		Zma001239	U	U	KM670444	[55]	U	N	
		OsMYB3R-2	MYBRS	<i>Dehydrin-responsive element-binding protein 2A, COR15A, RC12A</i>	BAD81765	[20]	U	<i>Arabidopsis</i>	Drought
		Rice	OsMYB4	U	Y11414	[71]	U	Apple	Drought, cold
MYBs		OsMYB48-1	MYBRS	e.g., <i>OsPP2C68, RAB2J, OsNCED4</i>	Os01g74410.2	[72]	D	Rice	Drought, salinity
		Wheat	TaMYB3R1	U	HQ236494	[73]	D	N	Drought, salt, cold
		TaMYB30-B	MYBRS	<i>rd29A, ERD1</i>	U	[7]	I	<i>Arabidopsis</i>	Drought
		Maize	ZmMYB-R1	U	JQ337942	[70]	D	N	Drought, salt, heat, cold
		OsWRKY3	U	U	Os03g55080	[81]	D	N	Drought
WRKY	Rice	OsWRKY4	U	U	Osg0g44010	[81]	U	N	Drought, cold, flood
		OsWRKY8	U	U	Osg1g02480	[81]	U	N	Drought, cold
		OsWRKY11	U	U	AK108745	[22]	U	Rice	Drought, heat
		OsWRKY18	U	U	Osg0g0440	[81]	U	N	Drought, cold, flood
		OsWRKY22	U	U	Osg1g61080	[81]	U	N	Drought, cold, flood
		OsWRKY24	U	U	Osg0g43650	[81, 82]	D	N	Drought, cold

TF	Species	TFs name	Cis-element recognition	Downstream genes	Accession/or locus number	Reference	ABA (D/I)	Transgenic plants	Stress inducible/tolerance
Rice	WRKY	OsWRKY42	U	U	Os04g21950	[81]	U	N	Drought, cold
		OsWRKY45	U	U	Os05g14370	[78]	D	<i>Arabidopsis</i>	Drought, disease
		OsWRKY50	U	U	Os02g26430	[81]	U	N	Drought, cold, flood
	WRKY	OsWRKY53	U	U	Os08g29660	[81]	U	N	Drought, cold, flood
		OsWRKY78	U	U	Os11g29870	[81]	U	N	Drought, cold, flood
		OsWRKY84	U	U	Os01g54600	[81]	U	N	Drought, cold, flood
Wheat	TaWRKY	OsWRKY96	U	U	Os01g14440	[81]	U	N	Drought, cold, flood
		OsWRKY100	U	U	Os09g16510	[81]	U	N	Drought, cold, flood
		TaWRKY2	U	STZ	EU663425	[82]	D	<i>Arabidopsis</i>	Drought, salt
	TaWRKY	TaWRKY10	U	Cor6.6, <i>rd28A, rd29B</i>	HQ703327	[83]	U	Tobacco	Drought, salinity
		TaWRKY19	U	e.g., <i>NfSOD, NtAPX, NtCAT</i>	EU663430	[82]	D	<i>Arabidopsis</i>	Drought, salt, freezing stress
		TaWRKY44	(TTGACC/TAAACC)	U	KR227395	[111]	U	Tobacco	Drought, salt, osmotic stresses
Barley	Hv-WRKY	Hv-WRKY38	U	U	CA060651	[133]	U	N	Drought
		OsbZIP23	ABRE	OsLEA3-1, et al.	AK072062	[23, 92]	D	Rice	Drought, salt
		OsbZIP12	ABRE	<i>LEA3, Rab16</i>	U	[34]	D	Rice	Drought
	Rice	OsbZIP16	ABRE	<i>LEA3-1, RAB16C</i>	Osb2g09830	[91]	D	Rice	Drought
		OsbZIP45	ABRE	U	Osb5g069300	[92]	D	Rice	Drought
		OsbZIP46	ABRE	e.g., <i>RAB21</i>	AK03188	[98]	D	Rice	Drought, heat, hydrogen peroxide
Wheat	OsbZIP	OsbZIP2/ RISBZ5	G-box	OsLEA3, <i>OstPP1, RAB25</i>	Osb6g5140	[24]	1	Rice	Drought, cold
		OsbZIP5/ OsbZIP71	ABRE or DRE	OsCAT, <i>OsNHX1, OsMY</i>	Osb9g13570	[25]	D	Rice	Drought, salt
		OsbZIP72	ABRE	LEA3, <i>Rab16</i>	Osb9g28310	[25, 93]	D	Rice	Drought
	TabZIP	TabZIP60	ABRE	e.g., <i>Atrd29A, ATRD20, Atrd29B</i>	KJ1562868, KJ1806555-KJ1806560	[84]	D	<i>Arabidopsis</i>	Drought, salt, freezing stress
		TaABP1	U	U	HQ166718	[134]	D	Tobacco	Drought, salt, cold
		HvbZIP13	U	U	U	[86]	D	N	Drought
Barley	HvbZIP	HvbZIP15	U	U	AK355261	[86]	D	N	Drought
		HvbZIP18	U	U	AK2515891	[86]	D	N	Drought
		HvbZIP20	U	U	AK359622.1	[86]	D	N	Drought
	HvbZIP	HvbZIP23	U	U	AK374525.1	[86]	D	N	Drought
		HvbZIP29	U	U	AK359391.1	[86]	D	N	Drought, cold
		HvbZIP34	U	U	AK355082.1	[86]	D	N	Drought
Maize	HvbZIP	HvbZIP40	U	U	AK249686.1	[86]	D	N	Drought
		HvbZIP42	U	U	AK388116.1	[86]	D	N	Drought
		HvbZIP49	U	U	AK39418.1	[86]	D	N	Drought
	ZmbZIP	HvbZIP52	U	U	AK372616.1	[86]	D	N	Drought
		HvbZIP53	U	U	AK359129.1	[86]	D	N	Drought
		HvbZIP77	U	U	U	[86]	D	N	Drought
Maize	ZmbZIP	ZmbZIP17	U	U	NM_001158672	[89]	D	N	Drought, heat, salt
		ZmbZIP37	U	U	GRMZM5G858197	[88]	U	N	Drought
		ZmbZIP72	ABRE	e.g., <i>rd29B, RAB18, HIS1-3</i>	HQ28839	[90]	D	<i>Arabidopsis</i>	Drought, salt, osmotic stress
	ZmbZIP	ZmbZIP74	U	U	GRMZM2G448607	[88]	U	N	Drought
		ZmbZIP12	U	U	GRMZM2G103647	[88]	U	N	Drought
		Oshox22	CAAT/G/C ATIG	U	AY124440	[27]	D	Rice	Drought, salt
HD-Zip	Rice	Oshox4	U	U	AF145728	[104]	U	Rice	<i>Arabidopsis</i>
	Maize	Zmhdz10	CAATAATIG	U	JX514832	[108]	D	Rice, <i>Arabidopsis</i>	Drought, salt

Table 1. Overview on the main cereal transcription factor family members involved in drought.

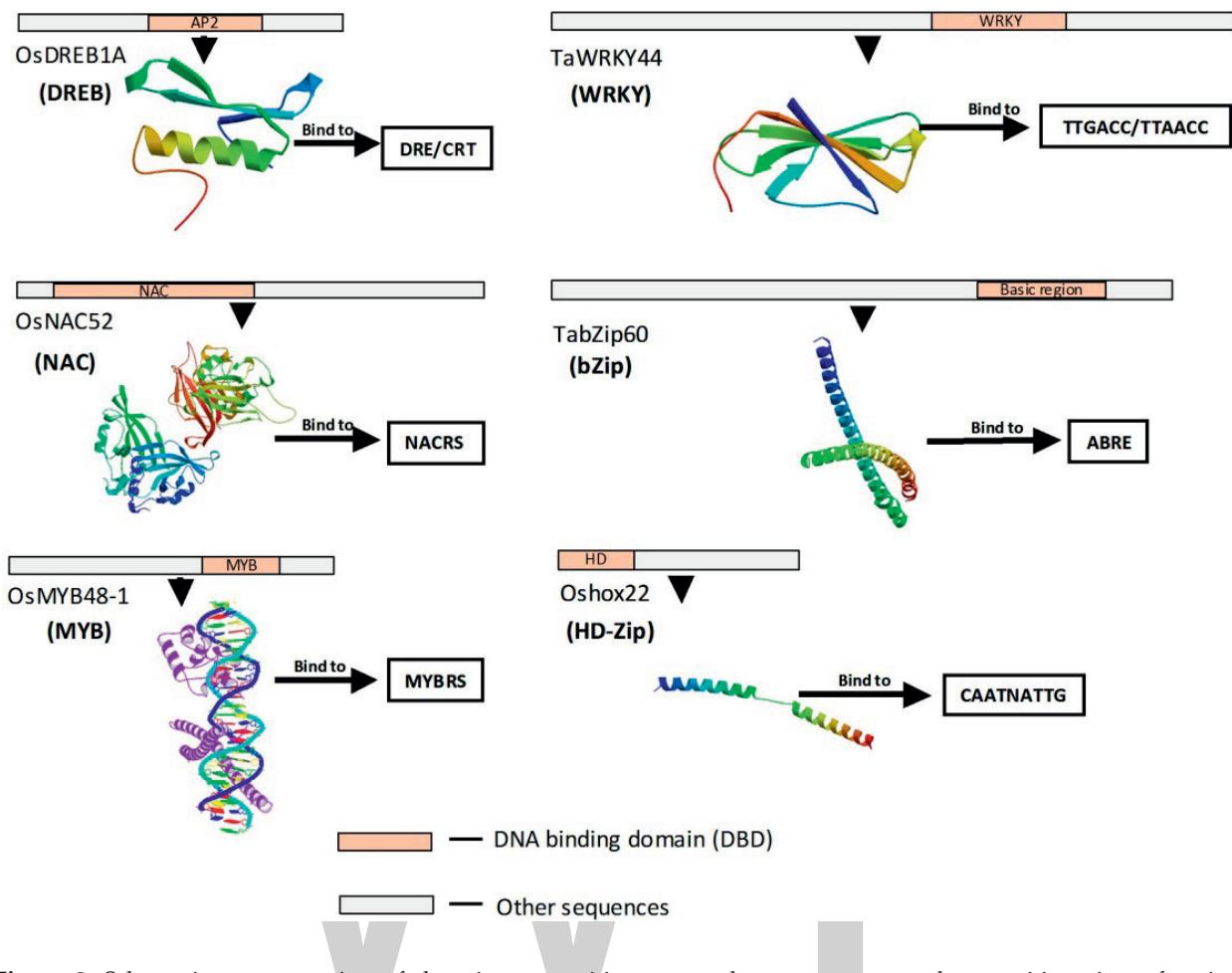


Figure 2. Schematic representation of domain compositions, secondary structures, and recognition sites of major drought-related TF families. The secondary structures were predicted using SWISS-MODEL (<http://swissmodel.expasy.org/>).

3.1. DREB/CBF family

The DREB/CBF family is a member of the AP2/EREBF superfamily of TFs, responsive to several stresses including drought [3, 8]. A cDNA encoding the first identified DREB/CBF family member CBF1 was isolated from *Arabidopsis thaliana* and characterized by Stockinger et al. [38]. DREB/CBF TFs possess about 60 amino acid long AP2 DBD which specifically recognizes a dehydration-responsive C-repeat (DRE/CRT) *cis*-element. The AP2 is a highly conserved domain of DREB family members. It contains two conserved motifs: the YRG and RAYD motifs. The YRG motif is considered to determine DNA binding and the RAYD motif, which forms an α -helix on the C-terminus, is supposed to play a role in PPI [39]. Drought responsive DREB TFs were also found in other plant species such as *Brassica napus* [40], *Triticum aestivum* [41], *Atriplex hortensis* [17], and *Oryza sativa* [42].

Many reported drought-inducible cereal DREBs were shown to be regulators improving stress endurance. In wheat, the gene *TaDREB1* [41] was induced by drought, salt, and cold. The transgenic barely containing *TaDREB2* and *TaDREB3* [13] showed improved tolerance in drought and low temperature conditions. In rice, 13 transcriptional factors including seven

DREB1 types (OsDREB1A, 1B, 1C, 1D, 1E, 1F, and 1G) and six DREB2 types (OsDREB2A, 2B, 2C, 2D, 2E, and OsAB14) [43] were isolated and analyzed. The overexpression of OsDREB1A [1] and OsDREB1F [3] resulted in transgenic *Arabidopsis* and rice plants with higher tolerance to salt, drought, and low temperature. OsDREB1G, 2A, and 2B were identified to be strong candidates in drought responsive pathways, while OsDREB1E could slightly improve the drought survival rate in transgenic rice [44, 45]. In different wheat cultivars, TaDREB1 was demonstrated to be inducible by drought, salt, low temperature, and ABA [41]. TaDREB2 and TaDREB3 significantly improved frost and drought tolerance in transgenic barley and wheat [13]. In maize, ZmDREB1A [11], -2A [94], and ZmDREB2.7 [46] contributed to drought tolerance. In barley, the gene *HvDREB1* [47] was induced by drought, salt, and low temperature, while the constitutive expression of HvCBF4 [48] increased the survival rate of transgenic rice under drought.

3.2. NAC family

The NAM/ATAF/CUC (NAC) TFs contain a unique feature, a conserved N-terminus DBD and a dissimilar C-terminus regulatory domain, and they are spread across the plant kingdom [37]. The name NAC is an abbreviation of three genes designated as no apical meristem (*NAM*), *Arabidopsis* transcription activation factor (*ATAF*), and cup-shaped cotyledon (*CUC*), which encode proteins containing homologous sequences as the NAC domain [49, 50]. *NAM* isolated from petunia by Souer et al. [49] was the first gene demonstrated to encode a NAC protein, followed by the gene *CUC2* from *Arabidopsis* [50]. Mutation of both genes resulted in the absence of apical shoot meristems [51] and led to floral abnormalities such as the alteration of petal primordia positions during the development stage. These evidences show that the TFs *NAM* and *CUC* play important roles in shoot apical meristem formation and determine the organ primordia positions in the floral meristem [49, 50]. But in relation to abiotic stress, the wheat genes *TaNAC2* [52] and *TaNAC69* [53] were strongly expressed under water deficiency and salinity. *TaNAC67* was found to decrease the cell membrane instability, preventing water loss and enhancing other physiological processes that were considered to be responsive to drought, low temperature, and salt stress [54]. In maize, *ZmNAC052*, *Zma000584*, *Zma006493*, *Zma001259z* [55], and *ZmSNAC1* showed increased transcription levels under water deficiency, indicating their potential role in drought tolerance regulation. In rice, *SNAC1* [56], *OsNAC5* [57], -6 [58], -9 [59], and -10 [60] altered the root structure for plant adaptation during drought. Further, the overexpression of *OsNAC045* [19] and *OsNAC52* [61] induced ABA sensitivity and conferred drought resistance in transgenic rice and transgenic *Arabidopsis*, respectively.

3.3. MYBs family

MYB is a group of ancient TFs found in viruses [62] and eukaryotes such as plants, animals, and fungi [63]. The first gene (*c1*) identified to encode MYB in plants was from *Zea Mays* [64]. MYB TFs contain a conserved DBD called MYB domain characterized by one to three imperfect repeated amino acid sequences (R1, R2, and R3). Each repeat sequence has around 50–53 amino acids which form three α -helices [65]. These three α -helices form a helix-turn-helix structure

when interacting with DNA. The MYB TFs are mostly classified into three classes according to the number of the MYB domain repeats: R1-MYB, R2R3-MYB [66], and R1R2R3-MYB [67]. These TFs participate in responses to dehydration, salt, cold, and drought [20, 68, 69]. In maize, the ZmMYB-R1 was induced by ABA, drought, low temperature, high salt, and heat [70]. The overexpression of rice genes OsMYB3R-2 [20], OsMYB4 [71], and OsMYB48-1 [72] improved the adaptive response to drought and other stresses in transgenic plants. In wheat, TaMYB3R1 [73] and TaMYB30-B [7] were found to be potentially involved in drought adaptation.

3.4. WRKY family

WRKY proteins belong to the superfamily WRKY-GCM1 of zinc finger TFs [74]. They exist in numerous plant species [75] and were reported to be involved in several biotic and abiotic stress responses and developmental processes such as embryogenesis and leaf senescence [75]. WRKY family members contain a highly conserved WRKY domain with 60 amino acids comprised of two motifs. One is the conserved WRKYGQK motif on the N-terminus and the other one is a zinc-finger-like motif on the C-terminus [76]. There are three main groups in the WRKY family according to the different number of WRKY domains and the variable structure of the zinc-finger-like motif [76]. Group I has two WRKY domains, whereas groups II and III have one WRKY domain. The zinc finger motifs of the WRKY domain in groups I and II are the same, but different in group III [75]. The group II has been divided into five subgroups by Eulgem et al. [76], designated as IIa, IIb, IIc, IId, and IIe, according to the conserved motifs outside the WRKY domain. Within these five subgroups, Zhang et al. [77] distinguished another three new groups (2_a+2_b, 2_c, 2_d+2_e). Several drought-related WRKY TFs were found in rice, wheat, and barley [78–80]. In rice, OsWRKY3, -4, -8, -18, -22, -24, -42, -50, -53, -78, -84, -96, and -100 were found to be co-expressed in drought and cold stress, and some of them were even expressed in different organs of flooded plants [81]. OsWRKY11 was identified to be involved in drought and heat response [22]. OsWRKY45 was found to be sensitive to ABA and considered to play a role in stomatal closure to improve drought and salt tolerance [78]. In barely, HvWRKY38 was shown to have a function in drought and cold response [9]. In wheat, the overexpression of TaWRKY2, -19 [82], and -10 [83] led to improved drought and salt adaptation in transgenic plants.

3.5. bZIP family

The basic leucine zipper (bZIP) family is another big group of TFs involved in diverse functions such as hormone and sugar signaling and organ development [84]. bZIP proteins commonly have a basic region for DNA binding and a conserved leucine zipper motif [85]. These TFs specifically bind to a DNA sequence with a core *cis*-element ACGT-like TACGTA (A-box), GACGTC (C-box), CACGTG (G-box), and an ABA-responsive element (ABRE) [84]. Some bZIP members were identified to participate in transducing ABA-dependent stress signals and were named as ABRE binding proteins (AREBs) or ABRE binding factors (ABFs) [86]. Numerous bZIP proteins were demonstrated or predicted to be involved in abiotic stress response in cereal plants, e.g., 89 in rice [87], 171 in maize [88], and 141 in barley [86]. They were classified into 11 groups A, B, C, D, E, F, G, H, I, S, and U according to the phylogenetic trees and DNA

binding motif [86] in maize, rice, and barely. The overexpression of several cereal bZIP TFs was identified to be in response to drought stress. In barley, 11 HvZIP members were identified to be down- or up-regulated by drought [86]. In maize, ZmbZIP37, -17, and -112 showed high expression levels in drought stress conditions [88, 89] and the overexpression of ZmbZIP72 enhanced the drought tolerance in transgenic *Arabidopsis* [90]. In rice, OsZIP12 [34], -16 [91], -23, -45 [92], -71 [25], and -72 [93] play a positive role in drought tolerance through ABA signal, while OsZIP52 [24] and -46 [94] were suggested to be a negative regulator in water deficiency. In wheat, the gene encoding *TabZIP60* was highly induced by salt, cold, and ABA, and the overexpression of *TabZIP60* enhanced the drought and frost tolerance in transgenic *Arabidopsis* [84].

3.6. HD-Zip family

The homeodomain leucine zipper (HD-Zip) family is a group of proteins that are unique to the plant kingdom. All members of the HD-Zip family contain the combination of homeodomain (HD) [6] with a following leucine zipper (Zip or LZ). HD is a conserved protein domain containing a 60 amino acids sequence, which is present in all eukaryotic species [95]. HD is a folded structure with three helices, which are responsible for the specific protein-DNA interactions [95]. HD-Zip family proteins have been classified into HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV [96–100] according to different domain structure and functions. According to Chan et al. [101], HD-Zip I TFs have less conserved motifs than HD-Zip II, and the sequences of HD-Zip I outside the HD-Zip domain are quite different, whereas HD-Zip II TFs have several common sequences outside the HD-Zip domain. The HD-Zip III TFs have four additional amino acids on the conjunction of HD and LZ compared with other three subfamilies [102]. HD-Zip I TFs contain no lipid/sterol-binding domain, designated StAR-related lipid transfer (START) domain, which was found in HD-Zip III and HD-Zip IV TFs [102]. Hence, the special structural feature of HD-Zip I TFs is the presence of HD and leucine zipper and the absence of common sequences outside the HD-Zip domain [103].

In rice, the *HD-Zip I* subfamily has 14 members: *Oshox4-6*, *Oshox8*, *Oshox12-14*, *Oshox16*, and *Oshox20-25* [104]. Three of them, *OsHOX6*, *OsHOX22* and *OsHOX24*, are homologs of the ABA and abiotic stress-inducible genes *AtHB7* and *AtHB12* in *Arabidopsis* [105]. *OsHOX22* and *OsHOX24* have been identified to be involved in drought, cold, and ABA response. *OsHOX22* is strongly activated by high salinity and ABA, but it is weakly induced by frost [27]. Zhang et al. [27] found that the insertion of T-DNA into the *OsHOX22* promoter region led to a decreased gene expression level of *OsHOX22* and reduced ABA content, but improved drought and salt endurance of rice seedlings. The authors believe that *OsHOX22* is a negative regulator for stress response by regulating an ABA-mediated signal transduction pathway and ABA biosynthesis [27]. The *OsHOX24* promoter has shown strong activation by water deficiency and high salinity [106]. According to the results of Agalou et al. [104], *OsHOX22* and *OsHOX24* can be induced by drought in drought-sensitive and drought-resistant cultivars, whereas *OsHOX6* can only be induced in drought sensitive cultivars. Although the role of *OsHOX6* and *OsHOX24* TFs is still not clear, the homologs of these TFs, *AtHB7* and *AtHB12*, have been found to be involved in ABA modulation by regulating the protein phosphatase 2C

activation and an ABA receptor gene activity [107]. In short, TFs Oshox6, -22, and -24 are responsive to dry conditions, similarly as the *ATHB7* and *ATHB12*, and their involvement in drought response might have relevance for ABA synthesis regulation [104].

In maize, the HD-Zip TF Zmhdz10 was found to play an important role in drought response [108], and in wheat, only two HD-Zip TFs, TaHDZipI-1 and TaHDZipI-2, were reported so far [109]. However, there is no information about the function of wheat HD-Zip proteins in drought response.

4. TFs target gene network activated in response to drought

TFs are involved in target gene network regulation through their DBD interaction with different gene promoter *cis*-elements mediated by ABA-dependent or ABA-independent signal transduction pathway (see Figure 3).

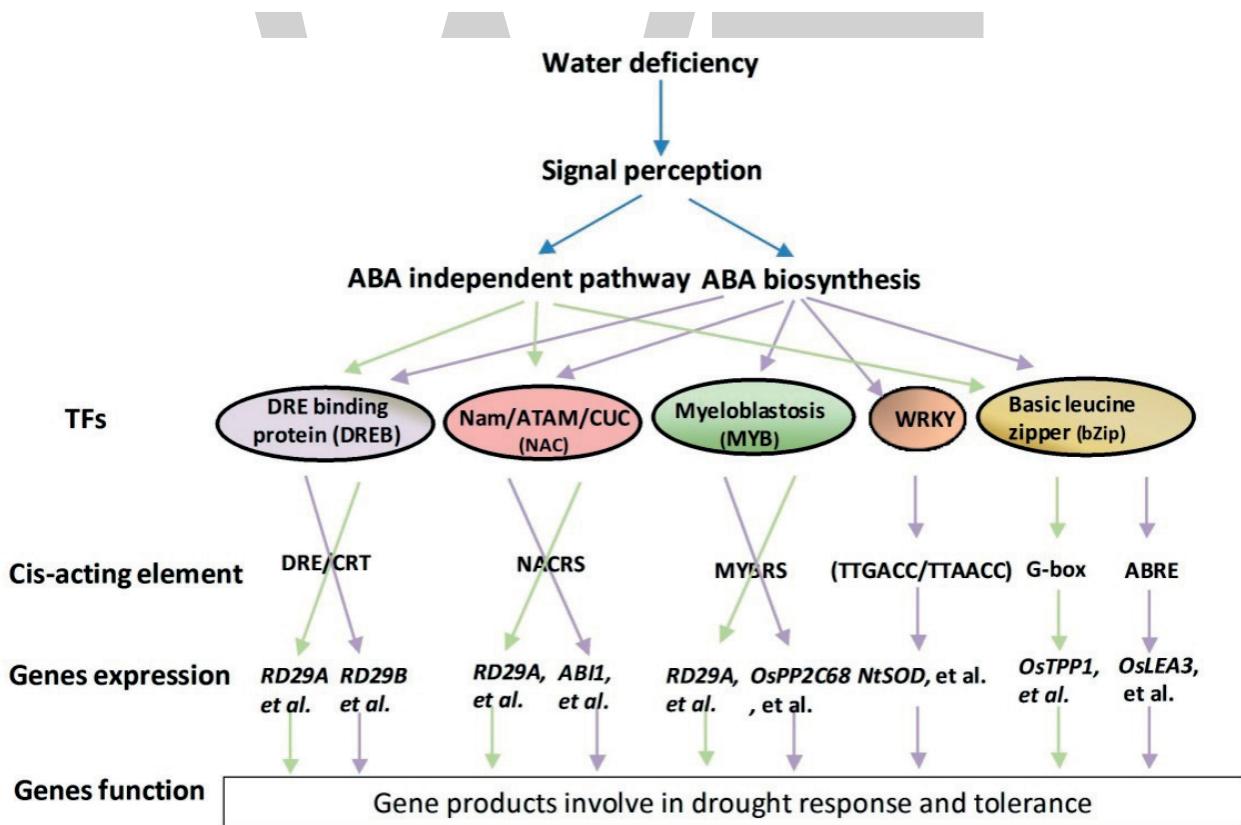


Figure 3. Cereal TFs target gene networks in response to drought through ABA-dependent and -independent pathways. The green arrows show the ABA-independent pathway and the purple arrows show the ABA-dependent pathway.

Most DREB family members such as OsDREB1A [1] and TaDREB1 [41] were found to be activated in ABA-independent pathways. They can enhance the stress tolerance by activating the expression of downstream genes such as late embryogenesis abundant (*LEA*) genes driven

by DRE/CRT *cis*-element. *LEA* genes, such as *COR15A* and *rd29A* (*Cor78*), are also designated as dehydrins (DHNs) or cold-responsive (COR) genes. Their expression products participate in cells protection from stresses by enhancing the membrane stability and correction of protein folding [13]. However, recent studies showed that some members of DREB also participate in ABA-dependent pathways. Wang et al. [3] found that the overexpression of *OsDREB1F* in transgenic rice results in the expression of ABA-induced genes *rd29B* and *RAB18*, whose promoters only contain an AREB element that cannot be recognized by DREB TFs. So far, there is no explanation on illuminating the way for *OsDREB1F* activating the expression of these two genes. The hypothesis is that *OsDREB1F* might interact with bZIP family members that can bind with an AREB element and modulate the transcription of *rd29B* and *RAB18* [3]. The same is true for *ZmDREB2A* [110] and the mechanism is still not clear. Besides, *ZmDREB2A* was identified to activate some downstream genes encoding detoxification enzymes that can protect cells from ROS. However, these genes do not contain a DRE/CRT element. Hence, it is possible that *ZmDREB2A* indirectly affects these genes' expression [110]. Besides, DREBs in different species might have different preference in regulating the expression of downstream genes with different core elements in their promoters. The rice *OsDREB1A*, e.g., prefers to interact with the CRT/DRE core element GCCGAC of genes such as *cor15A*, *rd29A*, and *rd17* instead of core element ACCGAC, while the *Arabidopsis* DREB1A and maize *ZmDREB1A* have equal competition for recognizing core element GCCGAC and core element ACCGAC in the downstream genes [1, 11].

In the WRKY family, more than 10 TFs isolated from rice were found to co-express under drought and cold stresses, but the downstream genes were still not determined [81]. TaWRKY44 from wheat was identified to recognize the core element (TTGACC/TTAACCC) in the promoter region of downstream genes and up-regulate genes encoding antioxidant enzymes such as NtSOD, stress-defensive proteins such as NtERD10C, and lipid-transfer proteins such as NtLTP1 to increase plants survival rate in drought. Thus, TF TaWRKY44 participates in regulating antioxidant enzyme activity and decreasing the ROS levels in order to prevent oxidative damage in plant cells [111].

Members of the MYB family regulate the expression level of different target genes involved in the ABA-dependent and independent pathways. The overexpression of *OsMYB48-1* was found to regulate genes such as *OsPP2C68*, *RAB21*, and *OsNCED4*, respectively, involving in ABA early signaling, late response, and the ABA synthesis pathway, contributing to increased drought tolerance under water deficiency [72]. In transgenic *Arabidopsis*, the overexpression of *OsMYB3R-2* increased the expression level of the downstream genes *DREB2A*, *COR15A*, and *RCI2A* and enhanced the plants adaptation to abiotic stresses [20]. Besides, TaMYB30-B was found to induce the expression of stress inducible genes *rd29A* and *ERD1*, involved in the ABA-independent pathway [7].

Members of the bZIP family were also found to regulate downstream gene transcription through the ABA-dependent and independent pathway. OsbZIP52 was suggested to bind to G-box *cis*-elements and down-regulate genes such as *OsLEA3* and *OsTPP* that can improve drought or cold tolerance in rice via the ABA-independent pathway [24]. The expression of

downstream genes *LEA3* and *Rab16* was activated by the transcription factor OsbZIP12 under water deficiency mediated by the ABA synthesis pathway [34].

Most TFs in the NAC family participate in the ABA synthesis pathway. OsNAC5 and OsNAC6 can recognize a core sequence (CACG) of the downstream gene *OsLEA3* and regulate the gene expression that changed root structure and resulted in higher drought tolerance through the ABA signaling pathway [35]. OsNAC6 also participates in up-regulating the transcription of genes encoding peroxidase, which can catalyze a series of oxidative reactions [33]. Some TFs even participate in both ABA-dependent and independent pathways. For example, TaNAC67 was found to up-regulate 10 abiotic stress responsive genes such as *rd29A* and *rd29B*, which were separately related to ABA-independent and -dependent pathways and four ABA synthesis/responsive genes such as *ABI1* [54], thereby improving stress tolerance in plants.

5. Homo- and hetero-dimerization of TFs

Homo- and hetero-dimerization of TFs plays an important role in certain cases and is considered as a pre-requisite for binding of DNA *cis*-elements. Formation of homo- and hetero-dimers plays a further function in modulating the DNA-binding specificity of TFs. Inability to form a dimeric complex may absolutely abolish the DNA binding ability of certain classes of TFs. The high complexity in the selection of hetero-dimerization partners and inability of some TFs to homo-dimerize but hetero-dimerize suggests that homo- and hetero-dimerization of TFs are not random processes, but that specific interactions between monomeric TFs forms are preferred. Hence, dimerization is likely to fulfill specific functions in gene regulation.

The dimerization ability of NAC proteins has been localized to the NAC domain [112, 113]. The residues in the highly conserved NAC domain are involved in the dimer contact and consist of hydrophobic interactions, a twisted anti parallel β -sheet sandwiched between two helices and two prominent salt bridges formed by the conserved arginine and glutamate [114, 115]. Experimental data suggest that NAC TFs are capable of forming both homo- and hetero-dimers. The NAC domain of NAC1 [116] and ANACO19 [113, 114] were shown to form homo-dimers. The NAC domains of OsNAC5 were shown to interact with the NAC domains of OsNAC5, OsNAC6, and SNAC1, generating both homo- and hetero-dimeric complexes. BnNAC14, a *Brassica napus* NAC protein, was shown to form hetero-dimers with BnNAC5-8, BnNAC485, and BnNAC3, but not homo-dimers. Mutational and deletion studies suggested that conserved NAC domains, in particular, the amino acids in close proximity to both the amino and carboxy-terminals, are necessary for mediating the formation of homo- or hetero-dimers [106, 112, 117].

Dimerization of the bZIP class of TFs is mediated by leucine zipper motifs, i.e., non-canonical repeats of leucine or other hydrophobic amino acid residues creating an amphipathic α -helix. The electrostatic attraction and repulsion of the polar residues situated next to the hydrophobic residues enables the formation and stabilization of dimers [118, 119]. Homo-dimeric rice OsbZIP71 is capable of exchanging its subunit to form hetero-dimers with members of the Group-C, in particular, with OsbZIP15, OsbZIP20, OsbZIP33, and OsbZIP88, suggesting a

possible role of hetero-dimerization in efficient binding to *cis*-elements on promoters of target genes [25]. A member of Group-A, the G-box-binding factor AtGBF4, interacts with the Group-G AtGBF1 and the Group-H AtGBF2. Similarly, the members of *Arabidopsis* Group-E, bZIP34 and bZIP61, form hetero-dimers with bZIP51 of Group-I and bZIP43 of Group-S, but none of these TFs belonging to Groups E and I can form homo-dimers due to electrostatic violations in the leucine zipper regions [15, 120, 121]. These data suggest that dimerization between members of within and between groups of bZIP TFs is highly specific and acts as a crucial mechanism to modulate the affinity for *cis*-elements and function of TFs.

In HD-Zips, the leucine zipper that is immediately downstream of the helical domain enables dimerization of HD-Zip TFs, which is a pre-requisite for DNA binding. The HD-Zip leucine zipper is a canonical repeat of leucine amino acid at every seventh residue creating an amphipathic α -helix, which forms a coiled coil structure during dimerization. Formation of hydrophobic interface and complementary charge interactions by the residues present in the coiled coil structure permit or inhibit the formation of dimers from monomeric HD-Zip TFs [105]. *In-vitro* studies have shown that dimerization of HD-Zip is a pre-requisite for DNA binding, and it is assumed that members of HD-Zip Class I and Class II families form hetero-dimers exclusively with other members of their own family [105, 122–124].

Though there is clear evidence for homo- and hetero-dimerization of WRKY proteins, the extent to which they form a functional dimer is unknown and yet to be determined. Of the seven WRKY subclasses, interaction between members of four WRKY class TFs have been experimentally demonstrated. In Group IIa WRKY TFs, dimerization is mediated by a canonical leucine zipper sequence, whereas in members belonging to other Group II and Group III subclasses, presence of leucine/isoleucine/valine residues at approximate seven-residue intervals at their N-termini form an amphipathic alpha helices similar to the secondary structure of a basic leucine zipper and mediate dimerization [125]. It is suggested that these potential leucine zipper sequences might mediate the formation of homo- and hetero-dimers within and between members of different subclasses of WRKY TFs. For example, *Arabidopsis* WRKY TFs belonging to the Group IIa, AtWRKY18, AtWRKY40, and AtWRKY60, form homo- and hetero-dimers [126]. Similarly AtWRKY30 interacted with AtWRKY53, AtWRKY54, and AtWRKY70 and formed hetero-dimers through leucine zipper motifs present at the N-termini of the subclass of WRKY TFs [127]. Interaction between different subclasses was observed in rice. OsWRKY71, a Group IIa WRKY TF, interacted not only with itself, but also with a Group IIId WRKY protein, OsWRKY51 [128]. Formation of homo- and hetero-dimer complexes between different WRKY TFs can have positive or negative effects on their DNA binding activities.

Formation of homo- and hetero-dimers offers an additional large combinatorial flexibility in the regulation of transcription. Performing an accurate analysis and developing a deeper understanding of roles of TFs in various biological processes will require the knowledge of other interacting partners, downstream genes, and location of expression in plant organs along with mechanism of homo- and hetero-dimerization of particular TFs. Thus, it may prove difficult to attempt to make informative conclusions about the roles of specific TFs on the basis of their singular overexpression without this level of knowledge.

6. Conclusions

TFs play a vital role in regulating gene transcription through different signal pathways to enable plants to adapt to harsh environments and abiotic stresses such as drought. Those TFs can recognize and interact with specific *cis*-elements of target genes via DBDs. Some TFs can up-/down-regulate downstream gene transcription, which encodes a subset of TFs integrated in plant hormone signaling pathways, forming a complex hierachic regulatory network. ABA, JA, and GA, the main plant hormones, act as key regulators in balancing plant growth and abiotic stress response. TFs, as the node of the cellular stress network and growth process, function as the interface of different phytohormone signal transduction pathways. A further layer of complexity is the formation of homo- and hetero-dimers, playing an important role in regulating DNA-binding specificity of TFs. These networks of signal pathways are regulating the activity of stress response TFs and other stress-relative genes, which in turn modulate physiological functions, such as stomatal movement, cuticle formation, and carbohydrate and lipid metabolism, to limit water loss and adapt to drought conditions. However, the hierarchy of TF interactions, the downstream genes' network, the interaction mechanism of the signal transduction pathways, and the protein–protein dimerization are not fully explored and still need more effort to be understood. More knowledge about plant protection system in hostile environments will help to find new tools for enhancing the plants to adaptat to abiotic stresses.

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Reactive Oxygen Species and Antioxidant Enzymes Involved in Plant Tolerance to Stress

Andréia Caverzan, Alice Casassola and Sandra Patussi Brammer

Abstract

Plants are continuously exposed to several stress factors in field, which affect their production. These environmental adversities generally induce the accumulation of reactive oxygen species (ROS), which can cause severe oxidative damage to plants. ROS are toxic molecules found in various subcellular compartments. The equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants. Due to advances in molecular approaches during the last decades, nowadays it is possible to develop economically important transgenic crops that have increased tolerance to stresses. This chapter discusses the oxidative stress and damage to plants. In addition, it reports the involvement of antioxidant enzymes in the tolerance of plants to various stresses.

Keywords: ROS, abiotic and biotic stress, oxidative stress, antioxidative mechanisms, tolerant plants

1. Introduction

Crop yield depends on the plant's ability to adapt to different types of environmental adversities, which generally induce oxidative stress. Environmental stress induces the accumulation of reactive oxygen species (ROS) in the cells of plants, which can cause severe oxidative damage to the plants, thus inhibiting growth and grain yield. ROS are involved in processes such as growth, development, response to biotic and abiotic environmental stimuli, programmed cell death, and may act as signal transducers. Stressors, hormones, development, and other several metabolic routes can stimulate ROS production that in turn may induce other routes or act directly as defense compounds [1].

Knowledge about the oxidative mechanisms in plants may contribute to the development of plants most well adapted to the environment and resistant to pathogens. Plants have defense mechanisms against oxidative damage that are activated during stress to regulate toxic levels of ROS. Antioxidant and nonantioxidant systems are involved in ROS detoxification.

During the last decades, antioxidant enzymes have been used to develop transgenic plants that have increased tolerance to several stresses. Therefore, this chapter will address the relation between abiotic and biotic stresses and ROS generation. The ROS production, major antioxidant enzymes involved in detoxification, and defense under stresses will be described. The involvement of the antioxidant enzymes in the tolerance of plants to various stresses will be also discussed.

2. Crop production and stress

Global agricultural production has tripled in the last 50 years because of increased demand due to population growth. Genetic breeding has improved crop yields per unit area. In 1960, the food requirement per capita was approximately 2,200 kcal/day. In 2009, the global food requirement per capita increased to more than 2,800 kcal/day. The global public spending on agricultural research and development rose markedly from 26.1 billion dollars in 2000 to 31.7 billion dollars in 2008; however, many challenges still remain in the agricultural sector [2].

Despite the efforts and progress achieved in recent decades in agriculture, growth and crop productivity are still negatively affected by several stress factors. Most crop plants grow in suboptimal environmental conditions, which prevent the plants from expressing their full genetic potential for development and reproduction, and consequently, these abnormal conditions lead to decreased plant productivity [3]. These stresses cause considerable production and economic losses worldwide.

Biological stress is an adverse force or condition that inhibits normal functioning of a plant [4]. These stresses may be biotic or abiotic. Biotic stresses include pathogens (viruses, bacteria, and fungi), insects, herbivores, and rodents. Abiotic stresses comprise cold (chilling and frost), heat (high temperature), salinity (salt), drought (water deficit condition), water excess (flooding), radiation (high-intensity ultra-violet and visible light), chemicals and pollutants (heavy metals, pesticides, and aerosols), oxidative stress (reactive oxygen species, ozone), wind (sand and dust particles in the wind) and soil nutrient deprivation [4, 5]. All of these factors may affect plant development and reproduction at different levels of severity.

Tolerance can be achieved by plant breeding or cultural practices that reduce losses, which is in turn accomplished by understanding the plant's response to its stressors and how they affect individual plants and plant processes [6]. Yield losses by oxidative damages occur because of an imbalance in plant synthesis and quenching. However, attributing this loss to the oxidative damage is difficult taking into account the several processes involved in ROS synthesis; however, stresses and oxidative damage are interlinked and are responsible for the yield losses [7] (Figure 1).

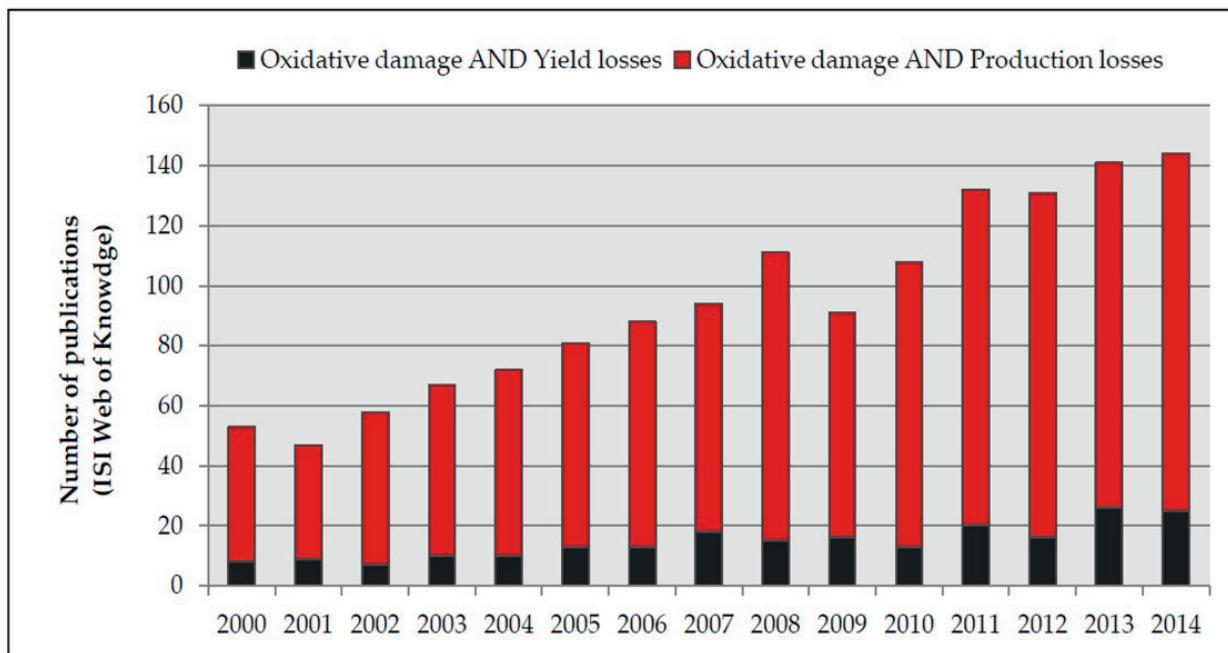


Figure 1. Evolution of the number of publications (2000-2014) addressing oxidative damage and yield/production losses. Total number of publications in 2000-2014 is 1,418. Source: ISI Web of Knowledge.

3. ROS generation

Environmental stress is directly correlated with increased accumulation of ROS. The equilibrium between production and scavenging of ROS may be disturbed by a number of biotic and abiotic factors, which may increase the intracellular levels of ROS [8]. When the level of ROS is increased and exceeds the defense mechanisms, the cell is in a state of oxidative stress [8, 9, 10, 11]. High concentrations of ROS are highly harmful to organisms, and when the symptoms persist, irreversible damage to the cells occurs, resulting in loss of physiological capacity and eventual cell death. Therefore, defense mechanisms against oxidative damage are activated during stress to regulate toxic levels of ROS [12] (Figure 2).

ROS are a group of free radicals, reactive molecules, and ions derived from oxygen. The most common ROS include singlet oxygen (${}^1\text{O}_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}\cdot$). These substances are highly reactive and toxic and can lead to oxidative destruction of the cell [8, 13]. ROS are found in various subcellular compartments such as chloroplasts, mitochondria, and peroxisomes due to the high metabolic activity that normally occurs in these compartments [13]. ROS are generated in chloroplasts via the Mehler reaction, in mitochondria via electron transport, and in peroxisomes via photorespiration.

The glycolate oxidase reaction, fatty acid β -oxidation, enzymatic reactions of flavin oxidases and disproportionation of O_2^- radicals are all metabolic processes responsible for the gener-

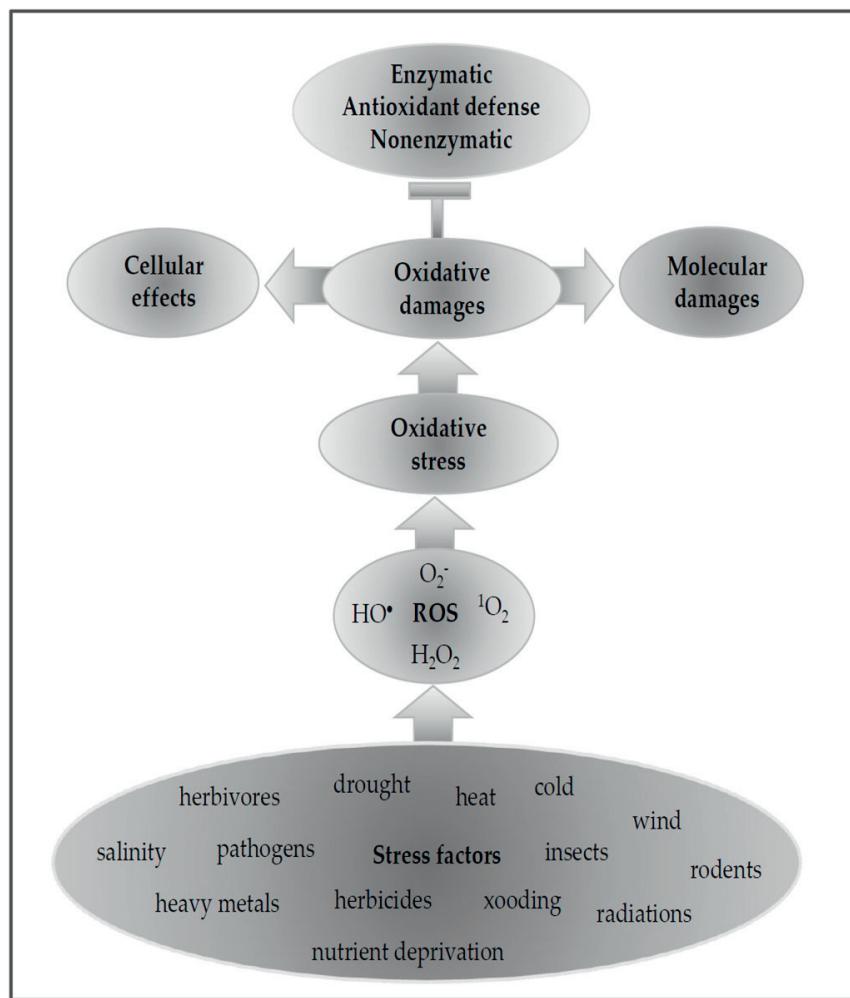


Figure 2. Stress factors, ROS generation, oxidative damage, and antioxidant defense. Several stress factors increased the ROS production, such as HO^\cdot , O_2^\cdot , ${}^1\text{O}_2$, and H_2O_2 . The increased ROS levels lead to oxidative stress. Consequently, oxidative damage at the molecular and cellular levels occurs. Defense mechanisms against oxidative stress are activated to neutralize toxic levels of ROS. Singlet oxygen (${}^1\text{O}_2$), superoxide radical (O_2^\cdot), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^\cdot).

ation of H_2O_2 in different types of peroxisomes [14]. Cytoplasm, plasma membrane, apoplasts, endoplasmic reticulum, and extracellular matrix are also sources of H_2O_2 . In the cytoplasm, the electron transport chain associated with the endoplasmic reticulum is the main source of $\text{H}_2\text{O}_2/\text{ROS}$ [11]. H_2O_2 generation can also be via enzymatic sources such as plasma-membrane-localized NADPH oxidases, amine oxidases, and cell wall peroxidases [15, 16]. Different organelles and cellular compartments possess potential targets for oxidative damage, as well as mechanisms for eliminating excess ROS. However, the balance between production and elimination of ROS can be severely disturbed by several biotic and abiotic stresses [9, 15]. These disturbances in the ROS equilibrium can lead to a rapid increase in intracellular ROS levels, which can cause significant damage to cell structures [17]. The redox homeostasis is the equilibrium between the production and scavenging of ROS; however, when ROS production overcomes the cellular scavenging capacity, there occurs an unbalancing of the cellular redox

homeostasis resulting in a rapid and transient excess of ROS, known as oxidative stress [11, 12]. Thus, the antioxidant defense imbalance disrupts metabolic activities [18], causing severe oxidative damages to cellular constituents, which can lead to loss of function and even cell death [12].

ROS may affect many cellular functions, for example, they can damage nucleic acids (oxidation of deoxyribose, strand breaks, removal/deletion of nucleotides, modification of bases, and cross-linked protein-DNA), lipids (breaking of the chain and increasing the fluidity and permeability of the membrane), and proteins (site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, alteration of the electric charge, inactivation of enzymes, and increasing the susceptibility of proteins to proteolysis) and can activate programmed cell death [10, 11].

The balance between production and elimination of ROS at the intracellular level must be tightly regulated and/or efficiently metabolized. This is necessary to avoid potential damage caused by ROS to cellular components as well as to maintain growth, metabolism, development, and overall productivity of plants. This equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants [13, 15].

In plants, the major ROS-scavenging pathway is the ascorbate–glutathione cycle (AsA-GSH) in chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes. This cycle plays a crucial role in controlling the level of ROS in these compartments [15]. The AsA-GSH cycle involves successive oxidation and reduction of ascorbate, glutathione, and NADPH catalyzed by ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [15, 18]. Thereby, the AsA-GSH cycle plays an important role in combating oxidative stress induced by environmental stress. Many components of the antioxidant system of plants are already well characterized into plant models, and disturbances or alterations in this system are an excellent strategy to investigate the different signaling pathways involving ROS.

3.1. Nonenzymatic antioxidants

Nonenzymatic antioxidants are found in all cellular compartments. These compounds may act directly in the detoxification of ROS and radicals, or they can reduce substrates for antioxidant enzymes [15]. Nonenzymatic components of the antioxidative defense system include the major cellular redox buffers ascorbate (AsA) and glutathione (GSH) as well as tocopherol, carotenoids, and phenolic compounds [10, 13, 18].

Ascorbate is found in organelles of most plant cell types and in the apoplast. AsA is a crucial component of the detoxification of ROS in the aqueous phase due to the ability to donate electrons in enzymatic and nonenzymatic reactions. AsA can directly eliminate O_2^- , $OH\cdot$, and 1O_2 , and thus reduce H_2O_2 to water via the ascorbate peroxidase reaction [19]. AsA is generally maintained in its reduced state by a set of NAD(P)H-dependent enzymes, including mono-dehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase [13, 20, 21]. Moreover, AsA is involved in the regulation of cell division, the progression of G1 to S

phase of the cell cycle and cell elongation, and it participates in multiple functions in photosynthesis [22].

Glutathione is oxidized by ROS to form oxidized glutathione (GSSG), which is present in all cellular compartments. Along with its oxidized form, GSSG, GSH maintains the redox balance in cellular compartments. Several studies indicate that GSH is involved in regulating gene expression and the cell cycle due to the properties of the GSH:GSSH pair [15]. The glutathione and AsA antioxidants are abundant and stable and have appropriate redox potential to interact with numerous components and pathways.

Tocopherols (α , β , γ , and δ) is a group of lipophilic antioxidants [11]. The α -tocopherol is the largest scavenger of peroxy radicals in lipid bilayers. The α -tocopherol present in the membrane of chloroplasts protects them against photooxidative damage [19].

Phenolic compounds are abundantly found in plant tissues, such as flavonoids, tannins, hydroxycinnamate esters, and lignin, and possess antioxidant properties [23].

3.2. Enzymatic antioxidants

Enzymatic components of the antioxidative defense system comprise several antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), guaiacol peroxidase (POX, EC 1.11.1.7), and peroxiredoxins (Prxs, EC 1.11.1.15), which catalyze ROS degradation, and enzymes of the ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX, EC 1.1.11.1), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.8.1.7), that regenerate soluble antioxidants [13, 15, 18, 24]. This antioxidant system plays an important role in the maintenance of cell homeostasis and in the antioxidant response in plants.

Superoxide dismutases are enzymes that catalyze the dismutation of O_2^- to H_2O_2 ; therefore, they constitute a frontline in the defense against ROS. These enzymes may be attached to a metal ion (Cu/Zn, Mn, Fe, and Ni); thus, they are classified according to their subcellular location and metal cofactor. SODs are present in many organisms, such as bacteria, yeast, animals, and plants. Plants have multiple genes encoding SODs that can be regulated by development, tissue-specific and environmental signals [10, 25].

Catalases are responsible for the removal of H_2O_2 by reducing H_2O_2 to $2H_2O$. CATs are largely, but not exclusively, localized to peroxisomes. Plants possess multiple CATs encoded by specific genes, which respond differentially to various stresses that are known to generate ROS [9, 10].

Ascorbate peroxidases are enzymes that play a key role in catalyzing the conversion of H_2O_2 into H_2O and use ascorbate as a specific electron donor. Plants have different APX isoforms that are distributed in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisomes, and the cytosol. The APX genes are differentially modulated by several abiotic stresses in plants [26, 27, 28]. The balance between SODs, CATs, and APXs is crucial for

determining the effective intracellular level of O_2^- and H_2O_2 , and changes in the balance of these appear to induce compensatory mechanisms [8, 9, 10].

Glutathione peroxidases are nonheme thiol peroxidases that catalyze the reduction of H_2O_2 or organic hydroperoxides to water. The GPX proteins have been identified in many life species [29]. In plants, the GPX proteins are localized to mitochondria, chloroplasts, and cytosol.

Peroxiredoxins are a family of thiol-specific antioxidant enzymes that are involved in cell defense and protection from oxidative damage. These enzymes are widely distributed in plant cells and are important proteins in chloroplast ROS detoxification [30]. The peroxiredoxins are a group of peroxidases that have reducing activity in their active sites via cysteine residues. These enzymes do not possess a prosthetic group and catalyze the reduction of H_2O_2 , peroxynitrite, and a wide variety of organic hydroperoxides to their corresponding alcohols [31].

Guaiacol peroxidases are involved in H_2O_2 detoxification. The POX proteins are heme-containing enzymes that belong to class III or the “secreted plant peroxidases.” These enzymes are able to undertake a second cyclic reaction, called the hydroxylic reaction, which is distinct from the peroxidative reaction. Due to the use of both cycles, class III peroxidases are known to participate in many different plant processes, from germination to senescence, auxin metabolism, cell wall elongation, and protection against pathogens [32].

Monodehydroascorbate reductase is a flavin adenine dinucleotide enzyme that catalyzes the regeneration of AsA from the monodehydroascorbate radical using NAD(P)H as an electron donor. Thereby, MDAR plays an important role in the plant antioxidant system by maintaining the AsA pool [24]. Isoforms of MDAR have been reported to be present in chloroplasts, the cytosol, peroxisomes, and mitochondria [33, 34].

Dehydroascorbate reductase is a thiol enzyme that maintains AsA in its reduced form. DHAR catalyzes the reduction of dehydroascorbate to AsA using GSH as a reducing substrate [18, 24]. It is present in various plant tissues, and its modulation activity has been reported in various plant species [35].

Glutathione reductase is an NAD(P)H-dependent enzyme. GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH); it is a key enzyme of the AsA-GSH cycle; it protects cells against oxidative damage; and it maintains adequate levels of reduced GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress [20].

The great increasing number of publications addressing APX, SOD, CAT, POX, GPX, Prxs, MDAR, DHAR, and GR enzymes are examples of positive responses to biotic and abiotic stresses by these enzymes. Over the past fourteen years, significant efforts have been made to understand plant antioxidant system mechanisms related to stresses, so the number of publications reporting antioxidant enzymes and biotic and abiotic stresses has increased substantially (Figure 3A and 3B, ISI Web of Knowledge database). These data show the relevance of studying these enzymes assisting in the understanding of its involvement with scavenging of cell toxic products in diverse species and the relation between oxidative stress and biological processes.

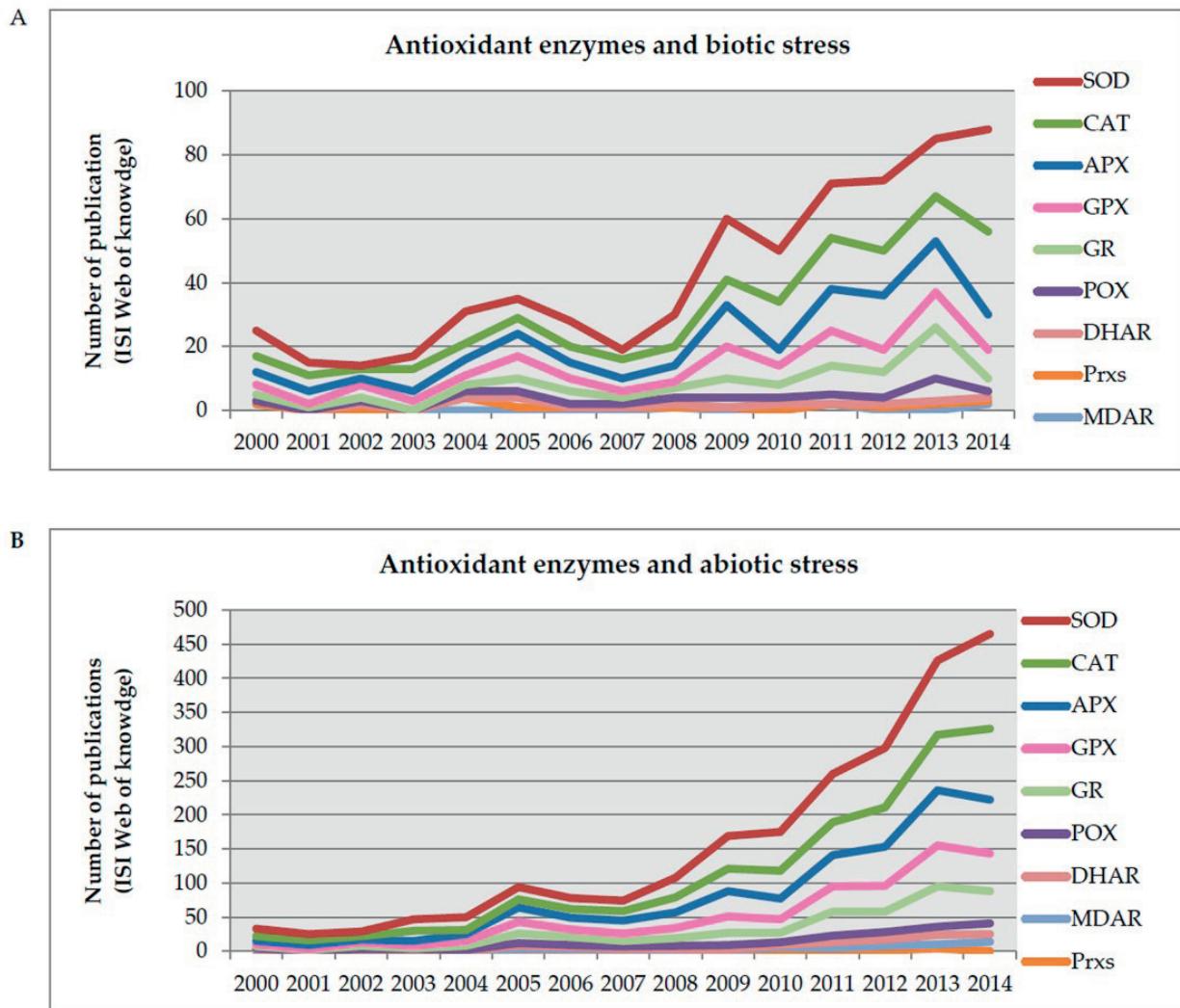


Figure 3. Evolution of the number of publications addressing antioxidant plant enzymes and biotic and abiotic stresses in the last fourteen years. (A) Data of the antioxidant enzymes and biotic stresses; (B) Data of the antioxidant enzymes and abiotic stresses. SOD (superoxide dismutase), APX (ascorbate peroxidase), CAT (catalase), GPX (glutathione peroxidase), POX (guaiacol peroxidase), Prxs (peroxiredoxins), MDAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GR (glutathione reductase) enzymes. Source: ISI Web of Knowledge.

4. Stress conditions and plants tolerant to stress

Stressful conditions are the main factor limiting agricultural productivity because plants do not reach their full genetic potential [4, 17]. Environmental conditions affect growth and development and trigger a series of morphological, physiological, biochemical, and molecular changes in plants. The metabolic pathways of plant organelles are sensitive to changes in environmental conditions [36]. Consequently, all environmental adversities have led to the world's agriculture facing serious challenges to meet demand. The increased consumption, allocation of land for other uses, and use of chemical products with implications for health safety are some examples these challenges [37].

The estimated world population for the year 2050 is nine billion people [2], and, consequently, the food demand will rise again. Therefore, it is necessary to increase the production and quality of food. Currently, the goal of many studies is the understanding of defense/tolerance mechanisms to different stresses in plants and to develop technologies and products that enable the generation of resistant/tolerant and more productive plants. Due to advances in molecular approaches, several crops of economic importance are being produced containing genes that encode stress tolerance using transformational technologies. Thus, several stress signaling and regulatory pathways have been elucidated and better understood.

Knowledge about the oxidative mechanisms in plants may contribute to the development of plants most well adapted to the environment. The maintenance of high antioxidant capacity to remove toxic levels of ROS has been related to increased stress tolerance of crop plants. Several studies show that maintaining a high level of antioxidant enzymes will help a plant to protect itself against oxidative damage by rapidly scavenging the toxic levels of ROS in its cells and restoring redox homeostasis.

Considerable progresses have been achieved in the development of plants tolerant to oxidative stress due to transgenic plants with altered levels of antioxidant genes to improve tolerance and productivity. This fact can be observed in Figure 4, which shows the increasing number of publications addressing antioxidant genes and its relation to tolerant plants in the last fourteen years (Figure 4). It highlights that SOD, CAT, and APX genes are the main antioxidant genes involved in the tolerance of plants to stresses, followed by GPX, GR, POX, POX, DHAR, MDAR, and Prxs, respectively. These studies reflect the importance and advances in comprehension of the antioxidant mechanisms and tolerance to stresses.

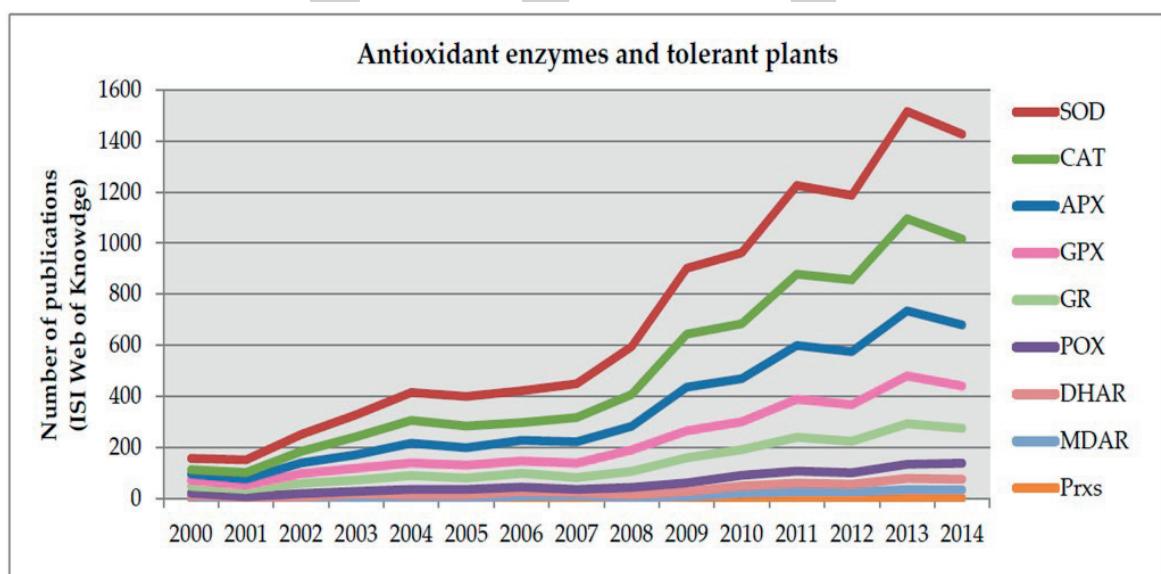


Figure 4. Evolution of the number of publications addressing antioxidant enzymes and plants tolerant to stresses in the last fourteen years. SOD (superoxide dismutase), APX (ascorbate peroxidase), CAT (catalase), GPX (glutathione peroxidase), POX (guaiacol peroxidase), Prxs (peroxiredoxins), MDAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GR (glutathione reductase) enzymes. Source: ISI Web of Knowledge.

Furthermore, the increased antioxidant activity has been reported to lead to better performance or tolerance response to several stresses. Using transgenic approaches, several species were studied aiming at the improvement of tolerance to stress enhancing antioxidant capacity of antioxidant genes. Table 1 shows some examples of the successful and positive responses obtained with regard to increased tolerance to cold, drought, heat, salt, hydrogen peroxide, methyl viologen, and metals stresses (Table 1). Improved tolerance using antioxidant genes are attributed by high antioxidant activity and more efficient ROS elimination. Plants expressing or overexpressing one or more antioxidant genes have more antioxidant capacity; consequently, plants can more efficiently eliminate excess ROS and protect their cellular components against toxic effects of ROS produced during the exposure to stress. As a consequence, plants suffer less oxidative injury and can tolerate a stress condition more effectively.

Gene	Native specie	Target specie	Stress tolerance	Reference
<i>Ascorbate peroxidase</i>	<i>Brassica campestris</i>	<i>Arabidopsis thaliana</i>	heat	[38]
	<i>Puccinellia tenuiflora</i>	<i>Arabidopsis thaliana</i>	salinity, hydrogen peroxide	[39]
	<i>Jatropha curcas</i>	<i>Nicotiana tabacum</i>	salinity	[40]
	<i>Hordeum vulgare</i>	<i>Arabidopsis thaliana</i>	zinc, cadmium	[41]
<i>Superoxide dismutase</i>	<i>Arachis hypogaea</i>	<i>Nicotiana tabacum</i>	salinity, drought	[42]
	<i>Tamarix androssowii</i>	<i>Populus davidiana x P. bolleana</i>	salinity	[43]
	<i>Pisum sativum</i>	<i>Oryza sativa</i>	drought	[44]
	<i>Oryza sativa</i>	<i>Nicotiana tabacum</i>	salinity, water, PEG-treatment	[45]
<i>Catalase</i>	<i>Brassica oleracea</i>	<i>Arabidopsis thaliana</i>	heat	[46]
	<i>Brassica juncea</i>	<i>Nicotiana tabacum</i>	cadmium	[47]
	<i>Triticum aestivum</i>	<i>Oryza sativa</i>	cold	[48]
<i>Glutathione peroxidase</i>	<i>Triticum aestivum</i>	<i>Arabidopsis thaliana</i>	salinity, hydrogen peroxide	[49]
<i>Peroxiredoxins</i>	<i>Solanum tuberosum</i>	<i>Solanum tuberosum</i>	heat, methyl viologen	[50]
	<i>Festuca arundinacea</i>	<i>Festuca arundinacea</i>	heat, methyl viologen	[51]
	<i>Suaeda salsa</i>	<i>Arabidopsis thaliana</i>	salinity, cold	[52]
<i>Monodehydroascorbate reductase</i>	<i>Malpighia glabra</i>	<i>Nicotiana tabacum</i>	salinity	[53]
	<i>Acanthus ebracteatus</i>	<i>Oryza sativa</i>	salinity	[54]
	<i>Avicennia marina</i>	<i>Nicotiana tabacum</i>	salinity	[55]

Gene	Native species	Target species	Stress tolerance	Reference
<i>Dehydroascorbate reductase</i>	<i>Oryza sativa</i>	<i>Oryza sativa</i>	salinity	[56]
<i>Gluthatione reductase</i>	<i>Brassica campestris</i>	<i>Nicotiana tabacum</i>	methyl viologen	[57]
<i>Ascorbate peroxidase/ Superoxide dismutase</i>	<i>Rheum austral/Potentilla astrisanguinea</i>	<i>Arabidopsis thaliana</i>	cold	[58]
	<i>Manihot esculenta</i>	<i>Manihot esculenta</i>	methyl viologen, hydrogen peroxide, cold	[59]
	<i>Solanum tuberosum</i>	<i>Solanum tuberosum</i>	heat, methyl viologen	[60]
<i>Catalase/Superoxide dismutase</i>	<i>Gossypium hirsutum</i>	<i>Gossypium hirsutum</i>	salinity, methyl viologen	[61]

Table 1. Some examples of the transgenic plants with potential stress tolerance expressing antioxidant genes

Some antioxidant enzymes such as SOD, CAT, APX, and GPX are better studied (Figure 3 and 4), but in general all enzymes have potential defense antioxidant activity helping in scavenging ROS in different ways, either by dismutation of O_2^{\bullet} to H_2O_2 , reduction of H_2O_2 , maintenance of the AsA pool, or of the adequate levels of GSH and GSSG, that all together maintain the antioxidant balance. In addition, antioxidant enzymes act in different subcellular compartments, thereby assisting in the ROS detoxification in organelles such as chloroplasts, mitochondria, peroxisomes, and in the cytosol. Besides, ROS-scavenging enzymes in various subcellular compartments might have a synergistic effect to improve stress tolerance in plants [59].

Many attempts aiming to increase the tolerance of plants to environmental stresses using antioxidant genes have been made by researchers. However, due to the great complexity of the antioxidant system and plant stress tolerance, we cannot state that ROS scavenging is the only factor that determines the level of tolerance, because other factors and several genes pathways are involved in the stress tolerance in plants. Furthermore, it must be emphasized that stresses often occur in combination; thus, the relation between ROS signaling mechanisms in different stress responses is very complex [62]. When under the effect of a combination of stresses, the plants respond differently than when experiencing just a unique type of stress [63]. Moreover, this can range depending on the plant species and cultivation area. Complexity of the tolerance mechanisms in plants is also a key factor because sometimes the alteration of one gene in the pathway can influence the expression of others, various genes and pathways being involved [64].

5. Conclusions

Plants activate antioxidant defense mechanisms under stresses, which helps in the maintenance of the structural integrity of the cell components and presumably alleviates oxidative

damage. Several antioxidant enzymes contribute to plant defense. The manipulation of ROS-scavenging enzyme systems is a worthwhile approach to produce transgenic plants with enhanced tolerance to a wide range of stress conditions; however, this needs to be further explored as many enzymes and isoforms can be involved, and ROS is only one of the potential parameters of plant tolerance against environmental variations and biotic stresses.

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Functional Genomics of Biotic and Abiotic Stresses in *Phaseolus vulgaris*

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Abstract

Common bean is the most important legume for human consumption in the world, being a crop extremely diverse in cultivation methods, uses, range of environments in which it is adapted, morphological variety, among others. Besides its high demand and production, this crop is threatened by a series of biotic and abiotic adversities during its life cycle, which leads to losses in yield of up to 100%. In this chapter, we explored the main constraints that affect common bean and the ways this plant reaches tolerance or resistance to them, highlighting studies at the molecular level that enabled to understand the mechanisms by which common bean perceives, responds, and adapts to a stress condition. Special focus has been given to the most recent findings in the understanding of the mechanisms underlying drought tolerance and anthracnose resistance. Thereby, we reviewed some genetic and functional genomic studies concerning the genes and pathways involved in each case. Furthermore, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

Keywords: Common bean, anthracnose, drought, genetic resources, gene expression

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important legume crop for consumption worldwide [1]. It is cultivated in a range of crop systems and environments, being Latin America the leading producer and consumer, where beans are a traditional and significant food source, especially in Brazil, Mexico, the Andean Zone, Central America, and the Caribbean [2]. As a source of protein, folic acid, dietary fiber, and complex carbohydrates, common

beans are considered nutritionally rich and when consumed as part of the diet can lead to an increase in the use of maize and rice proteins since their amino acids are complementary [2]. They are also a good non-meat source of iron, providing 23–30% of the daily recommended levels of this element in a regular adult diet [2–3].

In Latin America, Africa, and Asia, common bean is primarily a small farmer crop cultivated with few purchased inputs and is subject to a large amount of biological, edaphic, and climatic issues [2–4]. Conditions under which common beans are regularly cultivated in these regions are extremely variable [3], and such factors coupled with the highly specific local preferences for seed characteristics (size, shape, color) have been challenging to establishing the breeding strategies in accordance with what is needed.

Beans from these regions usually present low yielding [2], since they are frequently cultivated employing low to non-mechanized irrigation systems. Common bean is mostly grown in drought-prone areas, and long-term drought exposure periods seems to be a global and endemic threat affecting the majority of the production areas [4]. It has been observed that common bean is particularly susceptible to drought especially during the flowering and grain-filling stages (R5 and R8, respectively) [5, 6]. Moderate levels of water deficit usually lead to a reduction in plant biomass, lower seed number per pods, earlier maturation, lower seed yield and weight, and reduction in nitrogen fixation [7].

Not only abiotic factors but also several biotic constraints represent a significant threat to common bean cultivation. Fungi, bacteria, viruses, and nematodes cause a series of diseases, concurring for the death of some plants or even significant areas from whole plantations, causing a severe reduction in yield. Examples of such diseases are rust, white mold, anthracnose, root rots, bacterial blights (halo, yellow, common), powdery mildew, mosaic viruses, etc. Environmental conditions (temperature, soil moisture) and management practices (varieties, crop rotation, irrigation, and chemical control) may prevent the establishment of some diseases and reduce losses, but for some of them the most appropriate strategy for controlling consists on the development of resistant varieties and high-quality seeds.

This chapter is especially driven to describe the most recent developments in the understanding of the molecular mechanisms involved in drought tolerance and anthracnose resistance. In that purpose, we outline important genetic resources of *Phaseolus vulgaris*, as well as the technologies and methods used toward these findings.

2. Genetic resources

2.1. Center of origin and domestication of common bean

Beans belong to the Fabaceae family (Leguminosae, Papilionoidae) and genus *Phaseolus*. About 55 species of *Phaseolus* are described but only five are cultivated: *P. vulgaris*, *P. acutifolius*, *P. lunatus*, *P. polyanthus*, and *P. coccineus* [8].

P. vulgaris is naturally distributed in a wide area from northern Mexico to northeastern Argentina. High morphological diversity has been found among wild populations of *P.*

vulgaris from one to the other extreme of the geographical distribution of the species [9, 10]. This variability is observed in different leaf shapes, growth habits, flower colors but especially for seeds in terms of colors, shapes, and sizes [10]. This variability has also been observed at the molecular level, with several molecular marker studies such as with microsatellites [11–15], AFLP [14–16], and SNPs [17–20].

Several of these studies recognized two major ecogeographical gene pools of wild beans: Mesoamerican and Andean. However, the geographic structure of the wilds reveals more complexity, with an additional third pool between Peru and Ecuador, characterized by a particular storage seed protein, phaseoline type I [21, 22]. Further examinations showed wild populations from Colombia to be intermediates. A marked geographic structure in populations from the Mesoamerican pool has also been described [23, 24]. Originally, the population from northern Peru and Ecuador was considered an ancestral population from which *P. vulgaris* originated. From this core location, beans probably were spread north and south, resulting in the Mesoamerican and Andean pools, respectively [22, 25, 26].

Nevertheless, based on several studies [27–29], there has been a discussion over an alternative and older hypothesis which considers that ancestral beans were distributed through Mesoamerica. The high genetic diversity encountered within these gene pools has been used to support this hypothesis. Furthermore, the Mesoamerican origin of the common bean has been suggested based on sequence analysis of data from five small gene fragments [32]. A whole-genome comparison among 30 individuals from each Mesoamerican and Andean wild populations showed high genetic differentiation among gene pools and, a demographic inference for the Andean gene pools, suggested it was derived from a Mesoamerican population with only a few thousands of individuals [20]. Nevertheless, the debate on the origin of the species remains and more studies are on their way to better understand the core center of origin of common bean.

Likewise, the domestication process of *P. vulgaris* has been another matter of debate and extensive molecular studies. Initially, morphological and enzyme profiles showed the existence of two major centers of bean domestication: Mesoamerica and Andean, encompassing six races [10]. There are indications that nearly 8,000 years ago common bean was independently domesticated in Mexico and South America [30–33]. Domestication was followed by local adaptations resulting in landraces with different characteristics [20]. However, much more has yet to be deciphered and the recent application of genomic approaches is promising to a better understanding of the domestication processes of common bean and other crops [34].

2.2. Core collections

The high diversity of common bean has been collected in germplasm banks in which those are not only kept but also constantly improved, generating new genetic materials by adding new combinations obtained through many crosses and new generated populations. Several bean germplasm collections are available, but some of the core collections that must be highlighted here are held at the Centro Internacional de Agricultura Tropical (CIAT), in Cali, Colombia. Information on every wild and domesticated beans from this collection may be obtained in the website <http://isa.ciat.cgiar.org/urg/main.do?language=en>. Another core collection is from the

United States Department of Agriculture (USDA), found on <http://iapreview.ars.usda.gov>. Brazil has held a very significant collection of landraces and domesticated beans at EMBRAPA Arroz e Feijão and also at the Agronomic Institute of Campinas, which has been developing several new commercial varieties (<http://www.iac.sp.gov.br/areasdepesquisa/graos/feijao.php>). Much more details about bean collections are found on Genesys (<https://www.genesys-pgr.org/welcome>), a portal to information about Plant Genetic Resources for Food and Agriculture, describing many bean accessions and the places where they are kept. These collections comprise a very rich source of genetic materials that possess several features to be exploited in functional genomic and molecular breeding studies for the species. Among the genetic resources available are wild beans, landraces, breeding lines, recombinant inbred populations, all distinguished between the Andean and Mesoamerican gene pools.

2.3. *Phaseolus vulgaris* – The genome

A recent publication showed the work that has been done for many years to sequence the genome of the common bean, whose assembly has been made public by a consortium between the USDA-NIFA project “A sequence map of the common bean genome for bean improvement” and DOE-JGI and ARRA (*Phaseolus vulgaris* v1.0 – <http://phytozome.jgi.doe.gov/>). In total, 472.5 Mb of the 587-Mb genome were assembled and 98% of the sequence were genetically anchored on the 11 chromosomes, using a SNP high-density map (7,015 markers) genotyped in the RIL (recombinant inbred lines) population derived from the cross Stampede × Red Hawk and another map with 261 SSRs and a set of Infinium markers. The 472.5 Mb were arranged in 41,391 contigs (~9.32% gap) and the annotation revealed 27,197 total protein-coding genes and 31,638 protein-coding transcripts, resulting in 4,441 total alternatively spliced transcripts [23]. The publication of this genome opened a series of new resources for developing research in many fields such as the mechanisms involved in biotic and abiotic stresses in common bean.

3. Identification of genes involved with anthracnose resistance

The pathogenic system *Colletotrichum lindemuthianum*/*Phaseolus vulgaris* has been studied as a model for almost one century [36] and, its infection mechanisms and disease development were extensively studied in the 1980s [37, 38, 39]. This species of *Colletotrichum* is one of the most studied due to its economic importance, infection strategy [38], ease of *in vitro* cultivation [40], and availability of an efficient and reproducible transformation system [41]. As a model system for plant/fungi interaction, it can provide valuable information in several aspects, like plant defense responses, phytoalexins, fungal-degrading cell wall enzymes, differentiation of fungal infection structures.

The susceptible common bean cultivars establish an interaction of compatibility with this fungus, what allows the development of the anthracnose disease, strongly affecting production and yield of beans; furthermore, this fungus has great variability and many races identified [42, 43]. With this, the genetic resistance is an important way of disease control. Genetic studies

indicate that the common bean resistance to the anthracnose is related to multi-allelic loci [44, 45], which mostly comprise dominantly inherited genes denominated *Co* [45]. Bean cultivars resistant to anthracnose containing *Co* gene (s) respond to pathogen inoculation with an incompatible interaction. This interaction initiates with the pathogenic fungus inoculation, causing physiological variations and rapid changes in gene expression that activate defense responses in the host plant. Necrotrophic points, typical of a hypersensitive reaction (HR), occur at the infection site, resulting in a limited fungal growth. The HR, considered the primary response of the plant to the pathogen attack, is characterized by an oxidative burst due to the formation of reactive oxygen species (ROS) [46]. This initial plant response can be considered definitive in the determination of resistance to the pathogenic agent.

In the compatible interaction, the establishment of the pathogen in the plant tissue is aided by the production, by the fungus, of virulence effectors induced by the host [47, 48]. The life strategy adopted by the fungus (hemibiotrophic) make infected tissues remain without outward symptoms for up to three or more days [49, 50], and only after the entrance in the necrotrophic phase cause plant cell death and emergency of pathogenic lesions.

Despite the multi-allelic resistance already described for the common bean, new sources of resistance should always be searched due to the high variability among pathogen populations and occurrence of newly evolved virulent races. Furthermore, knowing the molecular pathways involved with the process of resistance in the plant can enable the transference of important genes to susceptible cultivars.

Common bean is not a species prone to be genetically transformed, although there is already a transgenic cultivar resistant to the Golden Mosaic Virus [51]. Furthermore, the genome of common bean was made available only recently, and reverse genetics through the use of mutant lines is still difficult due to few resources. Then, transcriptomic analysis appears as a suitable method to investigate the changes in gene expression in a plant under any kind of stress.

3.1. Gene expression profiles from an incompatible interaction

Studying gene expression profiles of incompatible interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum* may be an advantageous strategy to identify genes involved with anthracnose resistance because it can provide a direct answer about the potential modulations occurring in metabolic processes during an infection event with a resistance response by the host.

The first study devoted to generate a unigene data set of common bean using ESTs sequencing was described by [52], through the analysis of three EST libraries from the cultivar SEL 1308, consisting of 19-day-old trifoliolate leaves, 10-day-old stem shoots, and 13-day-old stem shoots inoculated with the race 73 of *C. lindemuthianum* in an incompatible interaction. At that time, a total of 5,255 ESTs were sequenced, 2,332 from inoculated stem shoots, with 1,583 unigenes assigned for this library. More recently, [53] used this database to select candidate genes based on the number of ESTs found per unigene (or tentative contig) in each library, to study expression profiles in temporal and spatial scales during fungus infection. Twelve genes were

chosen and tested in leaves, hypocotyls and epicotyls inoculated with *C. lindemuthianum* (Figure 1).

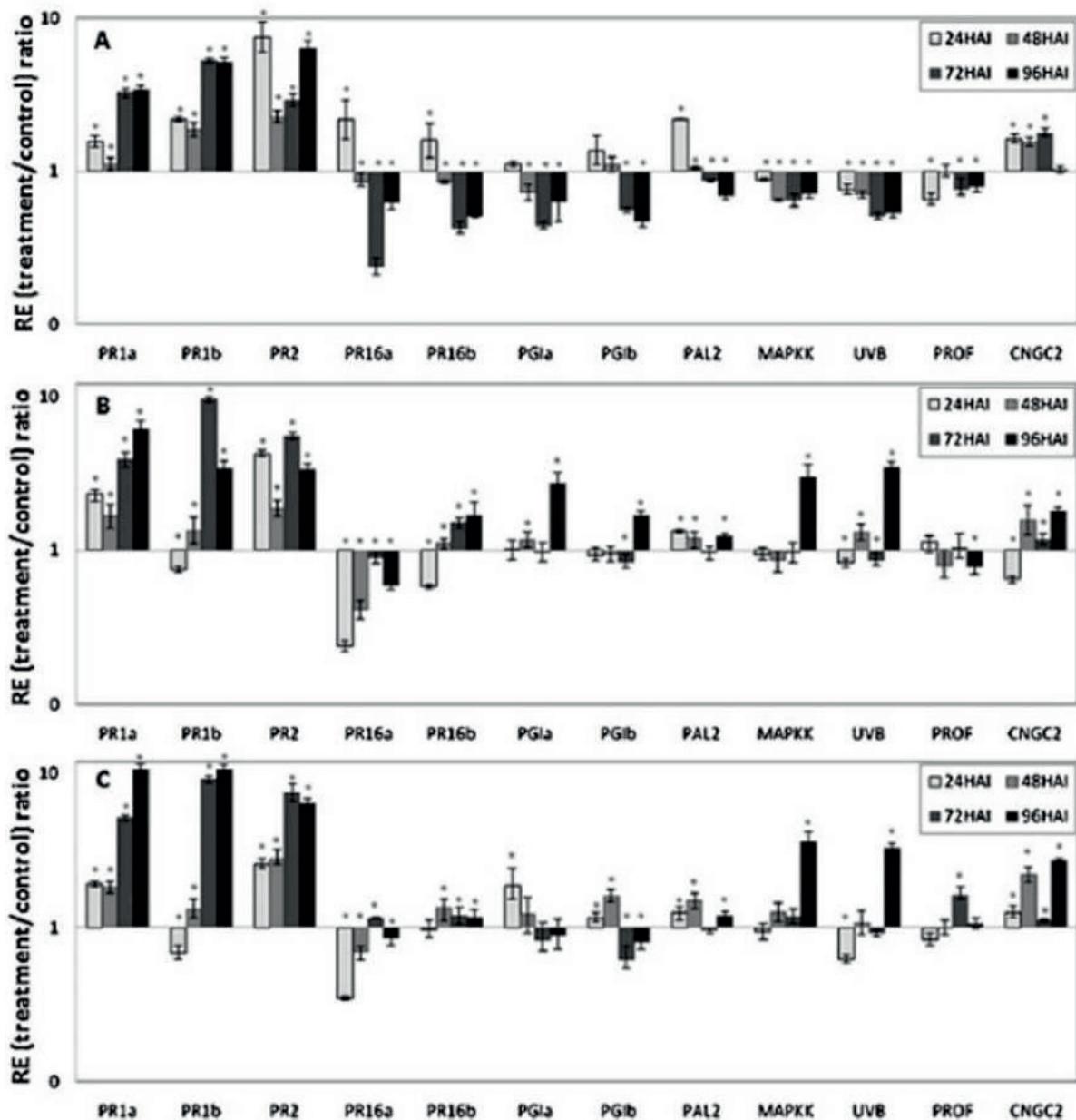


Figure 1. Relative expression (RE) ratio of 12 pathogenesis resistance-related transcripts in leaves (A), epicotyls (B), and hypocotyls (C) of common bean genotype SEL 1308 at 24, 48, 72, and 96 HAI with the race 73 of *C. lindemuthianum*. Non-inoculated tissue was used as control for expression levels to determine the RE ratio. The symbol * above the bars indicates statistical significance calculated using the Pair Wise Fixed Reallocation Randomization Test with $P \leq 0.05$. (Extracted from [53]).

All genes showed modulation during this incompatible interaction. Some of them were rapidly activated and kept this activation, like PR1a, PR1b (known as good molecular markers for SAR (systemic acquired resistance)), and PR2 (a b-1,3-glucanase) (Figure 1), which act in plant

defense by hydrolysing the cell walls of the fungal pathogens. All the others showed a variety of expression patterns according to time and tissue, for instance, PR16 proteins (germin-like), which were upregulated early in leaves and then fall down, and in epicotyls and hypocotyls only PR16b was upregulated in late periods of analysis (Figure 1). This kind of study not only give us an idea of the kinetics of induced defense responses of common bean against the anthracnose fungus but also can be used as a base line for others studies of resistance against a broad range of pathogens [53]. Furthermore, this work revealed differential and specific transcriptional profiles in different tissues of common bean, where specific defense processes may occur to contain the development of a pathogen. For more details, see [53].

3.2. The immune system model for *Phaseolus vulgaris/ Colletotrichum lindemuthianum*

The innate immunity is a primitive way of defense against microbial infection shared by plants, insects, and animals. Differently from mammals that have mobile cells specialized in defense, each plant cell is responsible for its own defense. Thus, each cell integrates environmental signals in order to activate local and systemic defense responses.

The same EST libraries described before [52] were used by [54] to investigate global changes in gene expression of *P. vulgaris* inoculated with *C. lindemuthianum* in an incompatible interaction. In an extensive bioinformatics analysis, the ESTs were aligned by tBLASTX with the *Arabidopsis thaliana* (L.) Heynh genome, which is completely annotated and curated. With this, it was possible to conduct a functional comparison between the fungus-inoculated and the mock-inoculated library. Figure 2 shows the overall mechanisms found in this study. It was found that some processes involved with plant-pathogen interaction were upregulated in common bean in response to the presence of fungus, like defense response to fungus (GO: 0050832), regulation of defense response (GO:0031347), regulation of response to stress (GO: 0080134), and stomatal movement (GO:0010118).

Response to cytokinin stimulus (GO:0009735) and ethylene-mediated signaling pathway (GO: 0009873) were upregulated, while jasmonic acid biosynthetic (GO:0031408) and metabolic (GO: 0009694) processes, as well as response to gibberellin stimulus (GO:0009739) and abscisic acid-mediated signaling pathway (GO:0009738) were downregulated, indicating that there may be a hormonal control and cross-talk in common bean defense against *C. lindemuthianum*. According to [54], hormonal mechanisms can be used in some pathosystems for resistance and in others for susceptibility depending on the fungus life-style. While jasmonates (JA) were found to be important in disease susceptibility in *Arabidopsis* and tomato infected with *Pseudomonas syringae* [55, 56], a biotrophic bacterium, in common bean it is not used in signaling since *C. lindemuthianum* is a hemibiotrophic pathogen.

Still based on the analysis of ESTs libraries, infected common beans have its metabolism modulated for detoxification from ROS burst, once HR is occurring during the incompatible interaction; also, a downregulation of genes was observed related to plant development (organelle fission (GO:0048285), cell cycle process (GO:0022402), pattern specification process (GO:0007389), post-embryonic morphogenesis (GO:0009886), and regulation of post-embryonic development (GO:0048580), typical of plants under stress that needs to reallocate resources to defense responses.

Finally, transcripts encoding for cell wall proteins showed an increase in abundance, suggesting that activities as cell wall modification, pathogen recognition, and transport and secretion of defense compounds are important in bean defense against anthracnose.

When looking for molecular components of the plant innate immunity (PTI – PAMP-triggered immunity or ETI – effector-triggered immunity), [54] observed that ETI (characterized by HR) can negatively regulate PTI. Transmembrane receptor protein tyrosine kinases and MAPKKK/MEKK transcripts were significantly downregulated in fungus-inoculated library and this data validate by RT-qPCR.

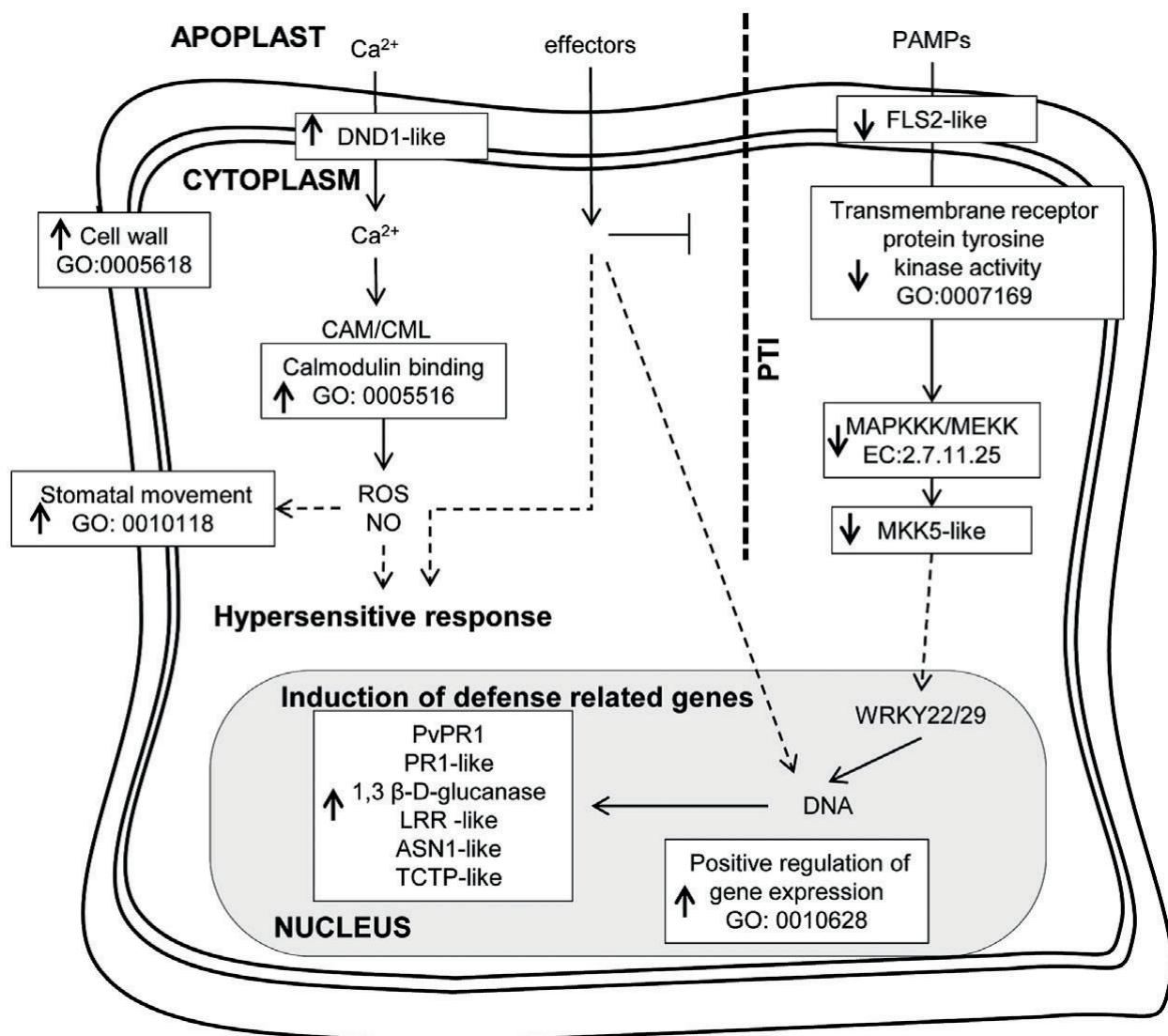


Figure 2. A model of the bean innate immune system. The proposed model represents key molecular components and metabolic processes known to be involved in plant-pathogen interactions. Gene Ontology (GO) categories and Enzyme Codes (EC) inside boxes are differentially represented in the bean EST libraries (arrowheads pointing down represent downregulation and arrowheads pointing up represent upregulation). Continuous arrows represent established relationship between components of the pathway and intermittent arrows represent undirected relationship. Components of PAMP-triggered immunity (PTI) are depicted to the right of the diagram. (Extracted from [54]).

4. Identification of genes involved with drought tolerance

4.1. Gene expression profiles from Subtractive Libraries of cDNA and RT-qPCR

Long-term global climate changes have conducted to an increase in the occurrence of drought episodes in different locations around the globe [57, 58]. This fact concurrently with agriculture expansion into marginal areas have led to increasing environmental instability, a limiting factor for crop yielding with potential negative impact on food stocks worldwide. This problem is especially aggravated by the rapid human population growth and consequent augmented food demand, especially in developing countries. Therefore, drought has been considered one of the main abiotic constraints that affect agriculture [59].

Plant responsiveness to drought stress can be affected by different factors; it mainly depends on the severity of the event, including the extension of the water-deficit period, and if the plant has already been exposed to a previous regime of acclimatization to this condition [60]. Acclimatization to drought results from a series of integrated events that comprehend the perception of the stress by the plant, translation of the signal, the regulation of the expression of specific genes, and the consequent shifts at metabolic level [61].

Drought perception often leads to a reduction in the photosynthetic rates of the plant, affecting its growth, which is directly related to shifts in carbon and nitrogen metabolism [62]. This reduction on the photosynthetic net is a result of a series of coordinated events such as stomatal closure and the reduction on photosynthetic enzymes activity [63, 64]. At cellular level, drought stress results in the accumulation of the chemically reactive molecules containing oxygen termed as ROS (reactive oxygen species), which ultimately can also drive to the oxidative stress of the photosynthetic apparatus [65, 66], thus ROS-efficient removal for avoid oxidative stress can be used as a measure for drought stress tolerance in plants [67]. These molecules act inside cells as secondary messengers involved in signaling transduction that leads to specific stress responses [65]. At molecular level, some specific sets of genes can undergo different processes of regulation of their expression (mainly through cycles of induction and repression of expression) determining new protein synthesis profiles, therefore changing their biological functions [61]. Several genes have been both collectively and individually implicated in drought stress response in plants, but the identification of which ones would be more useful for adoption at breeding and transformation approaches aiming the improvement of drought stress tolerance remains a great challenge [68, 69].

Strategies for plant transformation and genetic breeding usually focus on the transfer of a single or a small set of genes that can codify for specific biochemical pathways or for final targets of the signal transduction pathways that usually are controlled by constitutively active promoters [70]. These gene products protect the plant against the damages caused by drought stress and are divided into different classes: osmoprotectors (amino acids, dimethyl-sulfonyl compounds, mannitol, sorbitol, complex carbohydrates); enzymatic and non-enzymatic ROS scavengers; LEA proteins; heat-shock proteins; ion transporters; fatty acid desaturases; aquaporins; signaling components (homologous to histidine kinases, MAP kinases, Ca^{+2} -dependent protein kinases, protein phosphatases, Ca^{+2} sensors, inositol kinases); transcription

factors (EREBP/AP2, bZIP, ABRE, NAC, MYB); and growth regulators (ABA, cytokines, brassinosteroids) [60–71, 72].

At the transcriptional level, expressed sequence tags (EST) sequencing has been widely used to discover and identify genes potentially involved in drought stress response [73, 74]. Therefore, by using a great amount of transcriptome profiling methods, researchers are being able to contrast genotypes with different potential for drought tolerance, thus increasing the already large datasets of candidate genes for using in studies regarding the improvement of drought stress in plants.

Suppressive subtractive hybridization (SSH) method has been successfully used to construct cDNA libraries enriched in transcripts that are differentially expressed in target tissues, developmental stages, and specific treatments in various biological systems [74,75]. The SSH method [76] consists on the hybridization of one cDNA population (*tester* – sample whose genetic profile is of interest, e.g., drought-tolerant genotype), with an excess of cDNA from a control population (*driver* – usually drought-susceptible genotype or well-watered control), followed by the separation of the nonhybridized molecules (*target genes* – the ones of interest) from the hybridized ones (what is common for both samples). In this session, we are aiming to present some of the results obtained by our group during the construction of a SSH library contrasting populations of cDNAs extracted from root tissues of two common bean genotypes, BAT 477 (tester – drought-tolerant) and Carioca 80SH (driver – drought-susceptible), both submitted to a 192 hours of water-deficit regime at the R5 developmental stage [77].

The sequencing of the SSH library consisting of a BAT 477 cDNA population enriched for transcripts exclusively expressed by this drought-tolerant genotype under 192 hours of water-deficit generated 1,572 valid reads that were grouped into 189 contigs and 931 singletons (total of 1,120 unigenes). Public green plant EST databases (available at the National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>) and bioinformatics tools were used for initial trimming, clustering formation, gene annotation. Final functional annotation was achieved using the Gene Ontology Consortium database (<http://geneontology.org/>) combined to the CS model (*CombinedScheme*) developed by [78] (<http://www.biochem.ucl.ac.uk/~rison/FuncSchemes/>) (for further details on adopted bioinformatics tools and analysis specifications, see [77]).

Gene annotation based on homology search using the BLASTX tool and redundant sequences with E-value $\leq e-5$ generated putative information on 896 reads: 315 reads displayed similarity with sequences with not yet assigned putative or hypothetical functions, and 259 reads had good quality control but had no similarity with sequences available in public databases. Table 1 lists the most abundant contigs annotated via BLASTX tool and classified under the biological process that they might be involved in the plant. Final functional annotation classification of the 896 reads is summarized in Figure 3. The six main functional classes are described as follows: 1. Cellular Metabolism (Energy, Macro/ Micronutrients); 2. Biological Process (Cell Division, Regulation, Signaling, Cell Death, Signal Transduction, and Nuclear Cycling); 3. Transport of Compounds; 4. Structural Organization (Membrane, Cell Wall, Nucleus, Organelles, and Nodules); 5. Information Pathways (DNA, RNA, proteins, and transposons); and 6. Stress Response (Biotic and Abiotic Stresses).

Access code	Number of reads	GI number	Description/ Species	e-value
<i>Cellular Metabolism (Energy/Micro and Macromolecules)</i>				
Contig147	3	255579310	pyruvate decarboxylase, putative [Ricinus communis]	4e-80
Contig7	3	83283965	malate dehydrogenase-like protein [Solanum tuberosum]	e-171
Contig23	3	255638912	glyceraldehyde-3-phosphate dehydrogenase [Glycine Max]	e-119
Contig28	3	255540625	glutaredoxin-1, grx1, putative [Ricinus communis]	2e-40
<i>Biological Processes</i>				
Contig123	3	224094081	spliceosomal complex, [Populus trichocarpa]	3e-35
Contig171	3	75304713	Methionine adenosyltransferase, [Phaseolus lunatus]	1e-83
Contig79	4	156181612	S-adenosylmethionine decarboxylase [Phaseolus vulgaris]	3e-25
Contig127	4	75304713	Methionine adenosyltransferase, [Phaseolus lunatus]	5e-90
<i>Abiotic Stress Response</i>				
Contig74	4	42571665	interferon-related developmental regulator family protein [Arabidopsis thaliana]	6e-53
Contig105	3	192910730	light-inducible protein ATLS1, [Elaeis guineensis]	2e-30
Contig14	3	75708857	group 3 late embryogenesis abundant protein, [Phaseolus vulgaris]	6e-23
Contig61	3	806310	proline-rich protein, [Glycine max]	7e-18
Contig37	4	1732556	LEA5 [Glycine max]	3e-34
Contig97	4	1350522	LEA protein [Picea glauca]	3e-27
Contig24	9	1732556	LEA5 [Glycine max]	3e-34
<i>Biotic Stress Response</i>				
Contig3	3	184202203	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig3	3	184202203	isoflavone synthase 1 [Vigna unguiculata]	1e-85
Contig17	9	130835	PvPR2 [Phaseolus vulgaris]	1e-79
<i>Transport</i>				

Access code	Number of reads	GI number	Description/ Species	e-value
Contig164	3	616516061	plastidic phosphate translocator-like protein1 [Mesembryanthemum crystallinum]	1e-61
Contig80	4	255587991	cation:cation antiporter [Ricinus communis]	1e-39
Contig2	3	255552798	ATP binding protein, putative [Ricinus communis]	8e-30
Contig64	4	255637247	calcium ion binding [Glycine max]	2e-38
<i>Structural Organization (Membrane, Cell Wall, Nucleus, Nodulation and Organelle)</i>				
Contig142	3	255549412	Vesicle-associated membrane protein, putative [Ricinus communis]	8e-31
Contig137	3	146233385	abscisic acid ABA receptor [Populus trichocarpa]	1e-24
Contig148	3	194466205	putative L24 ribosomal protein [Arachis hypogaea]	2e-23
Contig11	5	255584772	histone h2a, putative [Ricinus communis]	2e-27
Contig19	3	570139001	NitaMp027 [Nicotiana tabacum]	6e-33
Contig83	4	306825451	ARF3 (ADP-Ribosylation factor 3) [Arabidopsis thaliana]	1e-59
<i>Information Pathways (Processing of DNA, RNA and proteins/ Transposons)</i>				
Contig154	3	187940303	NAC domain protein [Glycine max]	8e-84
Contig51	4	20138704	eIF-5A [Manihot esculenta]	7e-40
Contig52	4	255646048	transferase activity [Glycine max]	2e-58
Contig162	3	155212489	N3 protein [Glycine max]	1e-47
<i>Unclassified</i>				
Contig72	3	255626205	unknown [Glycine max]	3e-78
Contig87	3	255639776	unknown [Glycine max]	3e-71
Contig98	3	255647862	unknown [Glycine max]	8e-55
Contig145	3	255646578	unknown [Glycine max]	5e-47
Contig6	4	224101339	predicted protein [Populus trichocarpa]	5e-30
Contig64	4	255637247	unknown [Glycine max]	2e-38
Contig77	4	255637264	unknown [Glycine max]	2e-10
Contig82	6	255629893	unknown [Glycine max]	7e-27

Table 1. List of most abundant contigs containing the original ID of SSH library, number of reads assigned, NCBI identification number (GI) of the EST used for gene putative annotation inference, EST description and correspondent species, e-Values. ESTs are organized according to the functional class Biological Process [77].

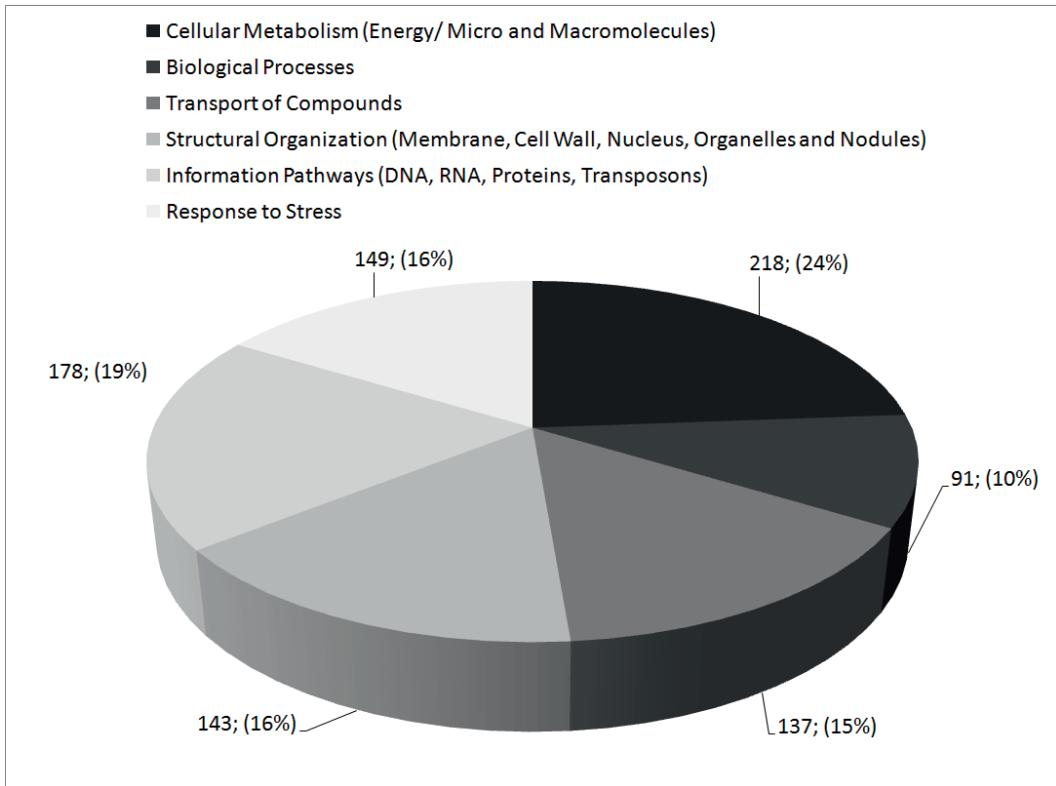


Figure 3. Final functional annotation classification of the 896 reads with positive putative gene description information ($E\text{-value} \leq e^{-5}$) obtained after blastx homology search using NCBI EST green plant public database (<http://www.ncbi.nlm.nih.gov/>). Each sector contains the relative EST numbers, in parenthesis the representation percentage in relation to the total number of ESTs successfully annotated. (Extracted from [77]).

The most abundant functional class was Cellular Metabolism (218 ESTs), something that was already expected since, as mentioned before, plants that undergo long periods of water deprivation tend to reduce its photosynthetic rates due to shifts in carbon and nitrogen metabolism, therefore needing to adjust its basal metabolic rates in order to keep homeostasis. Such elevated number of ESTs may be related to a more efficient mechanism of metabolic adjustment present in the drought-tolerant genotype BAT 477 that allows these plants to better adapt during the drought period, thus achieving better survival rates. And, 148 reads were grouped at the Response to Stress and some of them may be directly linked to drought stress tolerance: transcription factors (NAC, DREB, ABRE, WKRY, bZIP, MYB), transmembrane transporters like aquaporins, K^+/H^+ pumps and Ca^{2+} transporters, osmoregulators (LEA proteins, dehydrins, proline-rich peptide chains), and proteins associated with protection (heat-shock proteins, chaperones) and degradation (ubiquitins) [77].

A common bias usually associated with the SSH library construction technique combined with the traditional Sanger-based sequencing technique [79] is the possibility of obtaining false-positives. Recently, the use of SSH library technique combined with new high-throughput NGS-sequencing technologies [74–80, 81] has provided evidence for solving this issue since they are more able to achieve sample saturation. In RNA-Seq technologies, saturation could be reached when an increment in the number of reads does not result in additional true

expressed transcripts being detected or in more features called as differentially expressed when two or more conditions are compared [82]. However, the elevated costs usually associated with NGS-sequencing technologies make further experiment validation a more attractive option for researchers. The validation experiments consist of taking the same RNA samples initially used for cDNA library construction and re-analyzing them using a complementary technique, usually microarrays (for those species who already have this platforms available) [83,84] or RT-qPCR (quantitative reverse transcription PCR) [85].

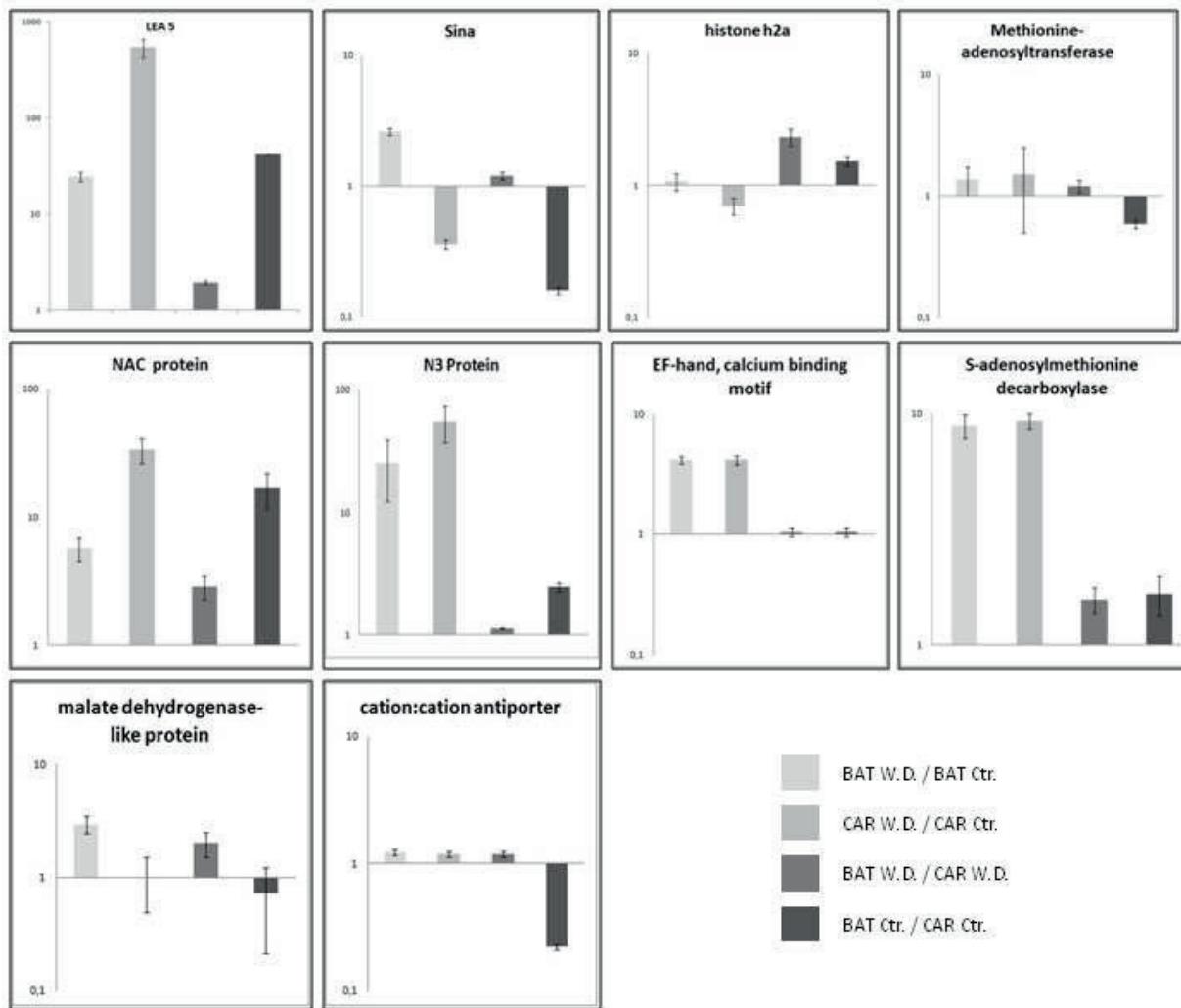


Figure 4. Relative gene expression profile of 10 ESTs selected from the BAT 477 drought stress-related SSH library [77]. Captions: BAT – BAT 477 drought-tolerant common bean genotype; CAR – Carioca 80 SH drought-susceptible common bean genotype; W.D. – 192h of water-deficit treatment; ctr. – control plants. BATWD/BATctr (BAT 477 under stress compared with BAT 477 control plants); CARWD/CARctr (Carioca 80SH under stress compared with Carioca 80SH control plants); BATWD/CARWD (BAT 477 under stress compared with Carioca 80SH under stress – SSH library initial condition); and BATctr/CARctr (BAT 477 control plants compared with Carioca 80SH control plants). (Extracted from [77]).

For the BAT 477 drought stress SSH library, it was selected as a set of 10 ESTs among those with most abundant contigs: *LEA5*, *Sina*, *histone h2a*, *methionine adenosyltransferase*, *NAC protein*,

N3 protein, EF-hand – calcium binding motif, S-adenosylmethionine decarboxylase, malate dehydrogenase-like protein, cation:cation antiporter. For each of the ESTs, a specific pair of primers for RT-qPCR analysis was designed [77] and gene relative expression quantification was obtained for the same tester and driver samples used for the SSH library construction (Figure 4). These results served well for the SSH library validation since all the selected transcripts revealed to be upregulated in BAT 477 plants under drought stress. Besides, for some of the transcripts (*LEA5, NAC protein, N3 protein, Ef-hand – calcium binding motif, and S-adenosylmethionine decarboxylase*), although they are expressed in lower concentrations on Carioca 80SH 192h drought-stressed plants, when compared to Carioca 80 SH controls, they undergo an even greater upregulation in relation to BAT 477 (Figure 4). This not only confirms the relevance of these transcripts on drought stress response regulation in common beans but also reveals that the drought-tolerant genotype BAT 477 may already keep a basal level expression of some important drought-related transcripts, thus stress perception by this drought-tolerant genotype may trigger more efficient signaling mechanisms that leads to a more discreet gene expression upregulation allowing the plant not to dislocate resources that otherwise may be saved for keeping homeostasis and therefore secure development and growth during the stress period.

4.2. DREB transcription factors as candidates for drought-tolerance improvement

Finding candidate genes and investigating their functional role and association with drought-tolerance traits and mechanisms have been of prime interest for many crop plants such as common bean. The DREB transcription factors subfamily has been studied in depth as candidate genes for breeding of abiotic stress tolerance. This group comprises a series of genes intermediating the regulation process to cope with abiotic stresses effects such as drought. They were originally described by [86], which identified a *cis*-acting regulatory element, DRE (dehydration responsive element), present in the gene promoter COR78/RD29A and involved in the response to drought, high salinity, and low temperature, further named as DREB (DRE-Binding). These proteins are capable of binding to DRE to activate the expression of genes of the stress signaling pathway. DREB transcription factors are unique to plant species and so far several genes have been described in *Arabidopsis* and other plants [87, 88].

The primary feature of a DREB transcription factor is the presence of a highly conserved protein domain, the EREBP/AP2. It was discovered within *APETALA2*, which plays an important role in flowering and seed development in *Arabidopsis*. Several proteins have been found containing this domain along their amino acid chain, consisting of a repeated motif of approximately 60 amino acids [89–91]. All these proteins are comprised in the larger superfamily EREBP/AP2 divided into three families referred as AP2, ERF, and RAV, based on their sequence similarity and the number of EREBP/AP2 domains [92]. The ERF protein family contains only one EREBP/AP2 domain and is subdivided into two main subfamilies, CBF/DREB and ERF [91]. The amino acids 14 and 19 of the EREBP/AP2 domain distinguish DREBs (valine and glutamic acid, respectively) from ERF (alanine and aspartic acid, respectively) [91]. In addition, ERF genes are involved primarily in responses to biotic stresses such as pathogenesis while DREB genes have main role in abiotic stresses responses.

DREB genes can be divided into six subgroups (A-1 to A-6). This categorization was based on phylogenetic trees as well as particular features related to their induction. The two most studied groups have been A-1 and A-2. Genes *DREB1/CBF* belong to subgroup A-1 and have been characterized as induced by low temperature in *Arabidopsis* [93], but other studies revealed some inducibility under drought and salinity as well [91, 94]. *DREB2* genes are primarily involved in responses to osmotic stress (dehydration and salinity) [91, 95].

Most of DREB findings have been associated with *Arabidopsis*; however, many studies have been performed with other species as well, revealing several new orthologs and different inducibilities for each one of the six DREB subgroups. Some of these findings have been done with legumes such as *Medicago truncatula* and *Glycine max*, close relatives to common bean.

Few studies have been published so far for common bean DREB genes, and they were mostly related to polymorphic sites identification along gene sequences. Ref. [96] categorized two orthologs *DREB2A* and *DREB2B* and identified polymorphisms between some Mesoamerican and Andean genotypes. Further investigation of these genes has been done to identify polymorphism patterns across wild and domesticated common beans. An attempt for phenotypic associations with drought-tolerance traits has been performed as well, but no clear patterns were obtained [18].

The research team of University of São Paulo, Brazil, has been studying DREB genes in depth. A pre-categorization study of the *PvDREB* gene subfamily has been done [97], showing putative DREB representatives for the species. Several genes have been isolated and their expression profiles determined under several abiotic stresses, including drought. One particular gene showed strong induction under many abiotic treatments, such as drought, salinity, and cold [98]. Some genes have been selected for a deeper molecular basis understanding as well as for their functional role in improving drought tolerance as well as other abiotic stresses.

Some other studies have found DREB genes in whole transcriptome profiles, such as in one experiment contrasting the drought-tolerant cultivar Long 22-0579 and the sensitive Naihua, in which a RNA-seq analysis was performed for samples under drought and control conditions. DREB transcription factors were identified to be differentially expressed and RT-qPCR analyses showed one transcript had the relative number of transcripts increased during the drought period [99]. Moreover, not only drought treatments have been analyzed but also one transcriptome profile has been done for a salt-tolerant bean cultivar named Ispir. It revealed several AP2/EREBP genes differentially expressed when contrasting a saline hydroponic solution with control conditions. Nevertheless, authors have not performed further categorization to identify which of those genes fitted *PvDREB*-specific characteristics [100].

Much more has to be done with DREB genes in common bean. Isolating and characterizing DREB genes for the species seems to be an important step toward the improvement of beans for abiotic stresses tolerance, especially for drought.

4.3. Phenotyping for drought tolerance in common bean

The identification of genomic regions or candidate genes, their functional role, and association with drought tolerance in common bean are fundamental aspects to understand the molecular signatures involved in acquiring such tolerance. However, in that purpose phenotyping methods are essential to effectively proving the effect of those genes on traits of interest. Thereby, it is important establishing and standardizing a phenotyping methodology to compare and select genotypes with different levels of stress tolerance in the studies one might be conducting. Furthermore, bringing data from the lab and greenhouse to the field is a big challenge, but of great importance for successfully applying the knowledge obtained about the genes, genotypes, and phenotypes of interest.

Phenotyping techniques have been developed to differentiating common bean accessions and cultivars for their levels of drought tolerance. Greenhouse trials have been applied to phenotype several shoot and roots traits and a common method employed has been the soil tube screening system assay that has been developed at CIAT [101]. Ref. [102] points out several traits that might be measured through such system, including many photosynthetic traits (photosynthetic efficiency, total chlorophyll content – SPAD, stomatal conductance, transpiration rates, leaf temperatures, leaf water potential), shoot and root biomass at the time of harvesting, leaf area and root traits (length, diameter, specific root length, and dry weight). Determination of root length might be done by image analysis system (WinRHIZO, Regent Instruments Inc.) [102] or might be manually determined by following root development on a graded plastic transparent tube in which plants were grown, all placed in PVC tubes.

The tube system developed by [101] was used to evaluate the effect of drought stress on root growth and distribution and compare different genotypes. Due to the difficulties of phenotyping roots in the field, this method has been shown to be a good complementary strategy applied in greenhouse conditions [102]. Examples in this sense are the studies of [103, 104] that analyzed the rooting patterns in greenhouse conditions with PVC soil cylinders and photosynthetic and yielding traits in different field areas. A population of recombinant inbred lines (RIL) from the crossing between the deep-rooting genotype BAT 477 and the small red-seeded and drought-susceptible DOR 364 was evaluated in both conditions. The greenhouse experiment showed that BAT 477 had significant larger root system based on root volume and deeper rooting ability, larger and thicker root, wide root diameter and biomass, under well-watered and progressive drought stress treatments [103].

For experiments conducted at the field, several traits can be evaluated since initial plant growth still harvesting. Ref. [102] made a very elaborated list with many parameters such as plant biomass at mid-pod filling and at harvesting time, seed yield, harvest index (HI), pod harvest index (PHI), drought intensity index (DII), and drought susceptibility index (DSI). The latter is based on the mean yields of a given genotype in drought stress and under no stress [102]. It assumes that one genotype will be more drought tolerant if the yielding is not so much reduced by the stress treatment in comparison to other genotypes. Pod harvest index has also been shown as a good indicator of drought tolerance, as shown by a field study in Ethiopia with the population from the crossing SXB 405 (breeding line) × ICA-Bunsi (white pea bean). Sensitive

lines presented significant reduction on PHI while no differences were observed for the most resistant lines [105].

Despite the availability of traits that might be evaluated in field conditions, the environment turns out to be a critical component interfering with results from one site to another. Drought field trials performed with the RIL population of the crossing BAT 477 × DOR 364, previously referred to the greenhouse experiment, showed significant variability across four locations evaluated [104]. A QTL analysis associating the field traits to a previous set of molecular markers disposed in a linkage map [106] showed significant QTL–environment interactions. Therefore, determining if one cultivar is tolerant to drought does not necessarily mean it will respond well to all environments, in a sense that it must be tested in multiple environments to check for its performance.

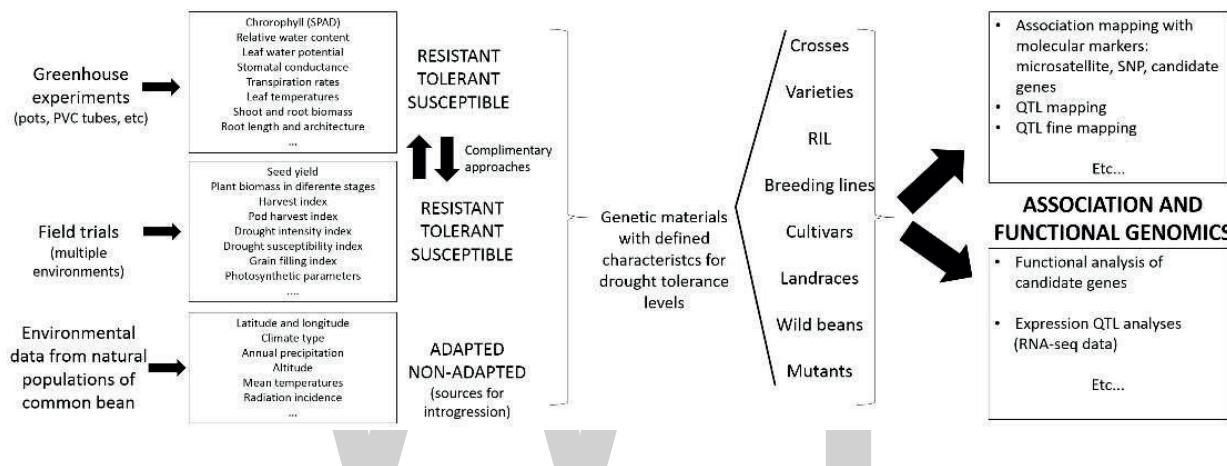


Figure 5. Strategies for phenotyping common beans for drought tolerance. Greenhouse experiments and field trials are complimentary strategies for evaluating several parameters and defining drought-tolerance levels. Recurring to wild beans based on environmental data might also be useful to select for beans adapted to drought episodes. Those evaluations altogether may be used toward the development of new varieties and the identification of genomic regions associated with the phenotypes evaluated as well as the functional role of candidate genes that are under characterization.

Although greenhouse and field methods have been developed to identify drought-tolerant genotypes and gene markers associated to such parameters, recent efforts have also been focused on the identification of sources of drought tolerance in wild beans spanning the natural area of distribution of *P. vulgaris* [107]. However, reliable estimations of drought tolerance in wild beans are not easy to establish, and attempts toward the development of new methods have been in course. Potential evapotranspiration models coupled with precipitation regimes were used to define a drought index for a series of wild bean accessions. Considering this factor along with the population structure might be a useful tool to analyze the levels of drought tolerance and use these materials for introgression of alleles of interest [107].

All these methods might be useful to carefully understand the phenotypic basis of drought tolerance variation in common bean genotypes. With standardized methods for the traits one might be interested, the accuracy between the association of molecular data and phenotypes might be much higher. It may be applied to QTL and association mapping studies, which link genome-wide molecular markers such as microsatellites, SNP, and gene-specific markers to

drought-related traits [103, 104, 106, 108]. On the other hand, standard greenhouse parameters can be used to test transgenic lines for determined candidate genes to verify their performances under imposed drought stress. Figure 5 shows a scheme of how greenhouse, field, and wild environment phenotyping studies might be useful for association and functional genomic studies in common bean.

5. Perspectives on the functional genomics of common bean

As mentioned before, common bean is not a species amenable for genetic transformation with the aim to test genes and to do functional studies. Thus, genomic mapping, transcriptomic and proteomic studies in contrasting genotypes, phases of development, different treatment/growth conditions, etc. are currently the most used approaches to identify genes linked to determined loci, verify changes in plant metabolism, and ultimately identify candidate genes suitable for molecular breeding or functional analyses.

The “omics” technologies and bioinformatics tools for large-scale data analysis have become essential to understanding the molecular systems that underlie various plant functions [109]. Despite common bean has been receiving increasing edible and economic importance, an investigation at a comprehensive omics level has been lacking in comparison to other model legume crops. As the genome sequences of *P. vulgaris* has become recently available, a new chapter has been opened for research with this crop. The genome release has provided a great miscellany of candidate genes that should be useful to improve common bean toward several different goals and approaches.

When considering abiotic stresses, some interesting NGS-related transcriptome data associated to drought [99] and salt-stress tolerance [100] as well as proteomic data related to drought [110], chilling [111], and osmotic stresses [112] have already been accessed. The consequential integration of a wide spectrum of omics data sets is then essential to promote translational research to engineer plant systems in response to the emerging demands of humanity.

Nevertheless, there is a big lack of information regarding interaction among stress sources. A recent trend for other crops has been the study of the effects of combined stress treatments such as drought versus salt, drought versus heating, drought × salt × nutrition, among others. These new studies try to represent most appropriately what really happens in the field, since plants are often subjected to multiple stresses. This should also be extended to the level of abiotic versus biotic stresses since many diseases are coupled with abiotic stresses at a certain stage of development of common bean. The available research on genomic, transcriptomic, and proteomic level on isolate stress-inductive factors should now be reunited in an attempt to elucidate the most complex phenomena involved in stress interactions. And, that should be extended to another level of complexity, which is establishing the interaction of both abiotic and biotic stress sources on common bean.

Regarding plant/pathogen interaction, until the moment the pathosystem *Phaseolus vulgaris*/ *Colletotrichum lindemuthianum* was only investigated in an incompatible interaction. However,

there are other combinations of genotype and pathogen races that lead to a compatible interaction and remain to be studied in order to compare these systems and understand which mechanisms are really responsible for the resistance.

Still, considering plant/pathogen interaction, in the past years, the LMD (laser micro-dissection) technology has been applied to study individual cells of plant-infected tissue and/or pathogen structures. This is because the way plant tissues were collected to do quantitative analyzes, as transcriptomic and proteomic, could generate a dilution of those cells in direct contact with the fungus into the whole tissue. This type of analysis allows a specific and localized evaluation. The LMD technique is based on the coordinated use of microscopy, laser and robotic, to localize, dissect, and capture cellular material [113]. This method has been important in selection and sampling of cells or cellular content in enough quantity and quality for DNA, RNA, protein, and metabolite analyzes, even in high throughput. Our group is employing this technology to study *P. vulgaris/C. lindemuthianum* interaction and *P. vulgaris*/mycorrhiza interaction under drought stress.

Looking for stress-resistance sources in other species and introgressing genes to common bean is another alternative for genomic improvement. A good example relies on the research that has been done for drought tolerance in common bean, based on interspecific crosses with other species of *Phaseolus*, such as tepary beans (*P. acutifolius*). They naturally span from the desert highlands of northwest Mexico to the southwest of the USA and thus they are good sources of drought, heat, and cold tolerance [114]. An interesting feature of tepary beans is their root system, which reveals extremely fine roots with rapid penetration in the soil with profuse branching, which enables quick access to limited soil water [115].

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All chapters in this book were first published in ABSPRAFP, by InTech Open; hereby published with permission under the Creative Commons Attribution License or equivalent. Every chapter published in this book has been scrutinized by our experts. Their significance has been extensively debated. The topics covered herein carry significant findings which will fuel the growth of the discipline. They may even be implemented as practical applications or may be referred to as a beginning point for another development.

The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

We would like to thank all the contributing authors for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

The editorial board has been involved in producing this book since its inception. They have spent rigorous hours researching and exploring the diverse topics which have resulted in the successful publishing of this book. They have passed on their knowledge of decades through this book. To expedite this challenging task, the publisher supported the team at every step. A small team of assistant editors was also appointed to further simplify the editing procedure and attain best results for the readers.

Apart from the editorial board, the designing team has also invested a significant amount of their time in understanding the subject and creating the most relevant covers. They scrutinized every image to scout for the most suitable representation of the subject and create an appropriate cover for the book.

The publishing team has been an ardent support to the editorial, designing and production team. Their endless efforts to recruit the best for this project, has resulted in the accomplishment of this book. They are a veteran in the field of academics and their pool of knowledge is as vast as their experience in printing. Their expertise and guidance has proved useful at every step. Their uncompromising quality standards have made this book an exceptional effort. Their encouragement from time to time has been an inspiration for everyone.

The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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