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REVIEW ARTICLE

Plant 14-3-3 proteins as spiders in a web of phosphorylation

Albertus H. de Boer · Paula J. M. van Kleeff · Jing Gao

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Abstract Protein phosphorylation is essential for many aspects of plant growth and development. To fully modulate the activity of specific proteins after phosphorylation, interaction with members of the 14-3-3 family is necessary. 14-3-3 Proteins are important for many processes because they "assist" a wide range of target proteins with divergent functions. In this review, we will describe how plant 14-3-3 proteins are as spiders in a web of phosphorylation: they act as sensors for phospho-motifs, they themselves are phosphorylated with unknown consequences and they have kinases as target, where some of these phosphorylate 14-3-3 binding motifs in other proteins. Two specific classes of 14-3-3 targets, protein kinases and transcription factors of the bZIP and basic helix-loop-helix-like families, with important and diverse functions in the plant as a whole will be discussed. An important question to be addressed in the near future is how the interaction with 14-3-3 proteins has diverged, both structurally and functionally, between different members of the same protein family, like the kinases and transcription factors.

Keywords 14-3-3 · Phosphorylation · Kinase · bZIP transcription factors · Flowering · Hormonal signaling

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A. H. de Boer (✉) · P. J. M. van Kleeff · J. Gao
Faculty of Earth & Life Sciences,
Department of Structural Biology, Vrije Universiteit,
De Boelelaan 1085,
1081 HV Amsterdam, Netherlands
e-mail: bert.de.boer@falw.vu.nl

Introduction

Protein phosphorylation is an essential post-translational modification in every day plant life that affects thousands of proteins. The process is reversible and can result in conformational changes causing proteins to alter their activity. All eukaryotic cells contain protein modules that act as a *sensor* for the phosphorylation status of specific phospho-sites. One such module is made-up by homo- or heterodimers of so-called 14-3-3 proteins. These 14-3-3 dimers not only act as sensors, but they also "translate" the phosphorylation event into a change in activity state of the target. Intriguingly, the translation of the Ser/Thr phosphorylation event is different for each target: i.e., it can result in activation/deactivation of enzymes, alter the translocation into/out of organelles like the nucleus, mitochondria and chloroplasts, it can prevent or stimulate proteolytic breakdown, act as scaffold for two proteins, block protein/protein interaction, etc.

So, what are 14-3-3 proteins? The basic properties of 14-3-3 proteins have been well described in a number of recent reviews (Aitken 2006, 2011; Gardino et al. 2006). Briefly, every eukaryotic organism tested so far has 14-3-3 encoding genes, and none has been found in prokaryotic genomes. Whereas animals typically have seven 14-3-3 genes, plants have more with, e.g., *Arabidopsis* having thirteen 14-3-3 genes in addition to two pseudo genes (Rosenquist et al. 2001). Crystal structures of plant 14-3-3 proteins have been resolved, showing dimers with a characteristic flattened horseshoe structure with a central cavity, containing two amphipathic grooves (Wurtele et al. 2003). The grooves are the main site for interaction between 14-3-3s and their phosphorylated targets. Based on their gene structure, plant 14-3-3s can be divided into epsilon and non-epsilon members. Most proteins interacting with 14-3-3s are phosphorylated on Ser or Thr residues present in a conserved binding motif and therefore 14-3-3 proteins can be considered

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phospho-serine/threonine sensors. Three canonical motifs have been defined so far: mode I (*R*/*K*)SX(*S*/*T*)^{P}XP, mode II (*R*/*R*)XΦX(*S*/*T*)^{P}XP (where *Φ* is an aromatic or aliphatic amino acid and *X* any amino acid) and a C-terminal mode III motif (*S*/*T*)$^{P}X_{1-2}$–COOH (Ganguly et al. 2005). It should be noted that with more target proteins available now, it is clear that some proteins also bind by means of a noncanonical, or even a non-phosphorylated motif (Aitken 2002; Sribar et al. 2003). Thus far, no enzymatic function has been ascribed to 14-3-3 proteins and their function seems to be solely due to physical interaction.

Although large scale 14-3-3 interactomics and mass spectrometry-based studies indicate that there might be hundreds of 14-3-3 target proteins in the plant, the interaction with only around 40 targets has been well characterized and described (Table S1). So, the challenge for the near future is to up-grade putative 14-3-3 targets as identified in the large-scale interactomics studies to "*gold-standard*" targets (Johnson et al. 2011). With more targets identified, we can start to address the question whether changes in the 14-3-3 phospho-proteome are interrelated, e.g., can we identify master kinases that phosphorylate multiple 14-3-3 targets in order to generate a coordinated response? In this way, we may also start to understand *when* (internal/external signals), *where* (subcellular compartments, part of the plant) and *why* (what are the consequences for the cellular functioning in the context of the whole plant) the phosphorylation driven interaction take place.

In this review, we will bring together information on plant 14-3-3 proteins that is rather scattered in the literature thus far. An overview of known plant 14-3-3 interaction motifs will give insight into the specificities of plant 14-3-3 motifs, which differ to some extent from those identified in animal 14-3-3 motifs (Chan et al. 2011; Johnson et al. 2010). We will highlight a neglected topic that must be important for the functioning of 14-3-3 proteins, namely the phosphorylation of 14-3-3 proteins themselves. Rather than giving a full overview of recent plant 14-3-3 literature, we will focus on the interaction of 14-3-3 proteins with two main classes of proteins, namely protein kinases and (bZIP) transcription factors. We will describe the characteristics of interaction, compare the effects on protein function, and discuss how they affect biological processes. In the end we, will list a number of questions that should be addressed in the near future to bring plant 14-3-3s to the central stage that it already has in animal biology.

Arabidopsis 14-3-3 isoforms and post-translational modifications

In *Arabidopsis*, 13 proteins are expressed and in analogy with their animal counterparts they are referred to with Greek letters. More recently, 14-3-3 genes were designated as General Regulatory Factors (GRF1-13) and both designations are used in the literature. Here, we will use the Greek letter designation and Table 1 can be used for conversion to the respective GRFs. Large parts of all 14-3-3 proteins, whether from yeast, plants, or animals, are evolutionarily conserved, where the conserved blocks make up the large amphipathic groove which acts as the docking site for phosphorylated target proteins. Based on this structural conservation and reports that plant and human 14-3-3 isoforms can complement the lethal *bmh1 bmh2* double disruption in yeast (Knetsch et al. 1997; van Heusden et al. 1996), it has been suggested that there is little isoform specificity. However, although there may be redundancy between closely related genes, it is now clear that there is functional specificity. Specificity arises at different levels: protein structure, cell-specific expression and differential transcriptional responses to internal (hormones) (Schoonheim et al. 2007b) or external (light, nutrients, temperature) clues (Denison et al. 2011). Isoform specificity may also arise from differential phosphorylation of 14-3-3 isoforms, what affects their cellular function in an isoform specific manner, as shown for animal 14-3-3 proteins (Aitken 2011).

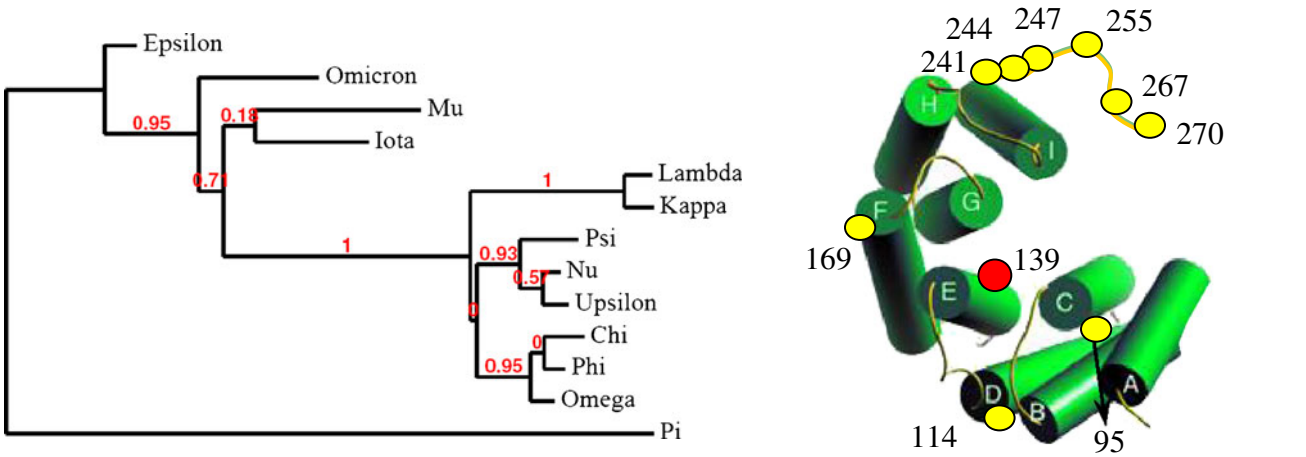
14-3-3 proteins are phospho-proteins

Phosphorylation of target proteins is the central theme in 14-3-3 biology. However, 14-3-3 proteins themselves are phosphorylated as well. From studies on animal 14-3-3s, it is clear that these phosphorylation events affect 14-3-3 function and because phosphorylation sites are not conserved among 14-3-3 isoforms, selective isoform regulation does occur (Aitken 2011). Following the sequence numbering of Hs14-3-3zeta, well-described phosphorylated residues in human 14-3-3s are S^{58}, S^{63}, S^{184}, and T^{232} (Aitken 2011; Obsilova et al. 2008). We will give a brief overview of how phosphorylation of these sites in animal 14-3-3s affect their function, whether these putative phospho-sites are conserved in plant 14-3-3 proteins and then the identified phospho-sites in plant 14-3-3s.

- S^{58} human 14-3-3: conserved in all *Arabidopsis* 14-3-3s, except in two closely related isoforms kappa and lambda (Table 1). Although S^{58} is masked in the dimer interface (formed by the two N-termini), this residue becomes accessible to protein kinase A after binding of cationic sphingolipids (Woodcock et al. 2010). Phosphorylation of S^{58} results in monomerization of the 14-3-3s, abolishment of the pro-survival signal of 14-3-3ζ and induction of cell death (Woodcock et al. 2010). The availability of sphingosine for 14-3-3 interaction is controlled by sphingosine kinase activity.
- S^{184} human 14-3-3: conserved in all plant 14-3-3s, except in 14-3-3mu. S^{184} is present in three human 14-3-3s and can be phosphorylated by mitogen-

Table 1 Annotation of *Arabidopsis* 14-3-3 proteins and the phosphorylation sites identified

14-3-3			Annotation	Phosphosties	95	114	169	241	244	247	255	267	270
Chi	χ	GRF1	AT4G09000	S95 [1], S267 [2, 3]	S	t	-	s	-	-	-	S	-
Omega	ω	GRF2	At1G78300	-	s	s	-	s	-	-	-	-	-
Psi	ψ	GRF3	AT5G38480	T162, S235 [2], T238 [3]	-	-	T	S	T	-	s	-	-
Phi	ϕ	GRF4	AT1G35160	S242 [4], S248 [3]	s	t	-	S	-	S	-	-	-
Upsilon	υ	GRF5	AT5G16050	S267 [3]	-	-	-	s	-	-	-	-	S
Lambda	λ	GRF6	AT5G10450	S112 [2]	s	S	-	s	-	-	-	-	-
Nu	ν	GRF7	AT3G02520	S251 [2]	-	s	-	s	-	-	S	t	-
Kappa	κ	GRF8	AT5G65430	S93 [1]	S	s	-	s	-	-	-	-	-
Mu	μ	GRF9	AT2G42590	S239 [3]	-	-	-	s	S	-	-	-	-
Epsilon	ε	GRF10	AT1G22300		-	-	-	s	-	-	-	-	-
Omicron	ο	GRF11	AT1G34760		t	-	s	s	-	-	-	-	-
Iota	ι	GRF12	AT1G26480		-	-	s	s	-	-	-	-	-
Pi	π	GRF13	AT1G78220		-	s	-	s	-	-	-	-	-
				Y137 maize = Y139 in chi									



Numbers refer to the position in the sequence of 14-3-3Chi, capital S or T indicates an identified phosphorylation site, lower scale s or t indicates that a phosphorylatable residue is present in the same position [1] Shin R et al. (2007) Phosphoproteomic identification of targets of the *Arabidopsis* sucrose nonfermenting-like kinase SnRK2.8 reveals a connection to metabolic processes. Proc Natl Acad Sci Unit States Am 104(15): 6460–6465; [2] Aryal UK, Krochko JE, and Ross ARS (2012) Identification of phosphoproteins in *Arabidopsis thaliana* leaves using polyethylene glycol fractionation, immobilized metal-ion affinity chromatography, two-dimensional gel electrophoresis and mass spectrometry. Proteonomics Res J 11 (1): 425–437. [3] Reiland S et al. (2009) Large-scale *Arabidopsis* phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiology 150(2): 889–903; [4] Benschop JJ et al. (2007) Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. Mol Cell Proteomics 6(7): 1198–1214

activated kinases (MAPKs) upon exposure to environmental stress. In phosphorylated form, the 14-3-3s dissociate from the pro-apoptotic proteins Bax and BAD and members of the forkhead transcription factors (Sunayama et al. 2005; Tsuruta et al. 2004).

– T/S^{232} human 14-3-3: not conserved in plant 14-3-3s. T/S^{232} is present in human 14-3-3 zeta and theta and can be phosphorylated by casein kinase1 and BCR kinase (Clokie et al. 2005; Dubois et al. 1997). This site is located in the flexible C-terminus involved in target binding and phosphorylation has an effect on interaction with targets like c-Raf and serotonin *N*-acetyltransferase (Clokie et al. 2005; Obsilova et al. 2004).

Plant 14-3-3 proteins have been reported to be phosphorylated on Ser, Thr, and Tyr residues (Aryal et al. 2012; Benschop et al. 2007; Giacometti et al. 2004; Shin et al. 2007). As presented in Table 1, the absence of identified phospho-sites in the non-epsilon group (except for one in 14-3-3mu) stands out. Site S^{95} in 14-3-3kappa and chi was found to be phosphorylated by SnRK2.8, a kinase that phosphorylates other targets related to energy metabolism such as glycolysis and carbon fixation (Shin et al. 2007). Phosphorylation of S^{95} may be an important determinant of isoform specificity since this residue is absent in the psi/upsilon/nu clade and the epsilon group. The expression of *SnRK2.8* is regulated diurnally, and a higher activity later in the

day may therefore affect the activity of 14-3-3 target proteins through 14-3-3 phosphorylation. A salt-induced calcium-dependent protein kinase, calcium-dependent protein kinases CPK3, also phosphorylates three 14-3-3s, namely omega, psi, and upsilon (Mehlmer et al. 2010); the phospho-sites were not determined. The majority of the identified phospho-sites reside in the flexible C-terminus, after the last α-helix (Table 1). Phosphorylation of these residues may have a large impact on 14-3-3 function because the C-terminal tail can act as auto-inhibitor (Shen et al. 2003) and determinant of specificity for target binding (Sinnige et al. 2005).

Although plants lack classic Tyr kinases that control Tyr phosphorylation in animals, available mass spectrometry data indicate that Tyr phosphorylation may be as extensive in plants as it is in animals (van Bentem and Hirt 2009). A maize 14-3-3 protein (GF14-6) was reported to be phosphorylated by IGFR-1 on Tyr^{137} (corresponding to Tyr^{139} in 14-3-3chi; Giacometti et al. 2004). This Tyr is conserved in all *Arabidopsis* 14-3-3s and the crystal structures show that it is located in the 14-3-3 binding groove and has the potential to negatively affect target binding through steric hindrance and/or charge repulsion. Indeed, binding of a phospho-peptide derived from the H^+-ATPase was reduced upon Tyr^{137} phosphorylation (Giacometti et al. 2004). In maize roots treated with the tyrosine phosphatase inhibitor phenylarsine oxide, the amount of 14-3-3 associated with the PM-ATPase decreased and reduced the Fusicoccin induced activation of the H^+-ATPase (Olivari et al. 2000).

Target phosphorylation that negatively affects 14-3-3 interaction

Whereas phosphorylation of a 14-3-3 interaction motif invariably increases the affinity of a target for 14-3-3 proteins, it should be kept in mind that there can be another level of control where phosphorylation of a Ser/Thr near the 14-3-3 motif reduces the affinity between target and 14-3-3. One example is the H^+-ATPase/14-3-3 interaction. Phosphorylation of the penultimate Thr residue (T^{947} in AHA2) by an as yet unidentified kinase strongly enhances the affinity for 14-3-3 proteins (Fuglsang et al. 1999). However, phosphorylation of Ser^{931} by the SOS2-like protein kinase PKS5 (CIPK11) reduces binding of 14-3-3 proteins to the C-terminus and decreases pump activity. The introduction of two phosphomimics in the ATPase C-terminus ($T^{931}D$ and $S^{938}D$) in PMA2 from tobacco, indeed prevented 14-3-3 protein binding even though the penultimate T^{955} was still phosphorylated (Duby et al. 2009). So, from this example, we can learn that phosphorylation driven 14-3-3/target interaction can be counteracted by phosphorylation of the target outside the 14-3-3 motif.

Plant 14-3-3 interaction motif characteristics and the 14-3-3 interactome

The "canonical" 14-3-3 interation motif

In a ground-breaking paper in 1997, Yaffe et al. defined for the first time two, so-called canonical, 14-3-3 binding motifs: mode-I, RSXpSXP and mode-II, RXY/FXpSXP, using phosphoserine-oriented peptide libraries (Yaffe et al. 1997). Later on, these motifs were further refined using screens for optimal binding of synthetic phospho-peptides adding preferred or negative amino acids for the X-residues (Obenauer et al. 2003) and used in Scansite to search for 14-3-3 motifs (http://scansite.mit.edu). More recently, a systematic approach using array-based peptides was used to further optimize prediction tools for 14-3-3 interaction motifs and generate new Scansite format matrices (Chan et al. 2011). All these studies were done with animal 14-3-3 proteins and the Scansite matrices defined may not be the optimal ones to find plant 14-3-3 interaction motifs. One reason is that a specific interaction motif must meet two requirements: it must accommodate the phosphorylating kinase and subsequently have good affinity for the 14-3-3 proteins. Chan et al. found for example that in animal 14-3-3 motifs an Arg at the +2 position (relative to the phospho-S/T) is as prominent as a Pro (Chan et al. 2011). The reason for this is probably that protein kinase C (PKC) has a preference for an Arg at +2. Plants do not have PKC kinases (Munnik and Testerink 2009) and that may be the reason that so far only one identified interaction site contains an R at +2 (Table S1; Kulma et al. 2004). In contrast, identified plant 14-3-3 motifs often have a Leu at the −5 position (Table S1), as pointed out by Johnson et al. (2010). The reason for this is probably also the preference of the phosphorylating kinases; in this case, members of the SnRK-family. Although accurate prediction of the target phospho-sites remains difficult, it can provide some guidance (in combination with other characteristics) when trying to identify 14-3-3 phospho-sites. In order to define a Scansite matrix for plant 14-3-3 motifs, we collected data from articles where 14-3-3 interaction motifs were identified, mainly through serine to alanine mutations that abrogated the 14-3-3 interaction. Based on these motifs, we generated a new Scansite PlantMatrix (Table S2) and a scan of validated 14-3-3 targets identifies in most cases the known site with the lowest score. In order to identify an interaction site in a putative target, three other criteria can be taken into account: (1) has the protein been found in large scale 14-3-3 interaction studies, (2) is the putative site known to be phosphorylated in vivo? (see PhosPhat, http://phosphat.mpimp-golm.mpg.de), and (3) is the putative site in a disordered region of the protein (http://www.disprot.org/pondr-fit.php?). The latter is relevant because analysis of a wide range of animal 14-3-3 interaction

sites showed that almost all 14-3-3-binding sites are inside disordered regions (Bustos and Iglesias 2006). In disordered regions, the protein adopts regular secondary structure but lacks fixed tertiary arrangement. Such disorder favors molecular recognition (e.g., by kinases) and protein–protein interactions. Moreover, it enables proteins to interact with numerous partners, what increases binding diversity (Bustos 2012). Disorder-to-order transition is suggested to occur upon interaction of the target with a 14-3-3 protein.

To test the above given criteria, we have taken Quercetin 3-O-methyltransferase OMT1 from *Arabidopsis* as example. OMT1 was identified as putative 14-3-3lambda target, but the interaction motif was not identified (Zhang et al. 1997). In Scansite, S^{96} and T^{83} yield the lowest score with the PlantMatrix, S^{96} is a reported phosphosite in PhosPhat, OMT1 was reported as 14-3-3kappa interactor in a 14-3-3 affinity study (Shin et al. 2011) and S^{96} is in a disordered peak. Based on these criteria, we predict that S^{96} is the phosphorylated residue in the 14-3-3 motif that determines the affinity of OMT1 for 14-3-3kappa/lambda.

The plant 14-3-3 phospho-interactome

The number of "*gold standard*" plant 14-3-3 binding proteins, a term coined in a recent comprehensive study on the mammalian 14-3-3 phospho-proteome (Johnson et al. 2011) is rather "limited" relative to the number of putative 14-3-3 binding proteins as identified in high-through-put studies. Such studies have been performed either as yeast two-hybrid screen of cDNA libraries, (Schoonheim et al. 2007a), pull-down studies using recombinant 14-3-3 proteins (Alexander and Morris 2006; Klychnikov et al. 2007; Schoonheim et al. 2007a; Shin et al. 2007; Shin et al. 2011; Swatek et al. 2011), tandem tag affinity using *Arabidopsis* plants expressing 14-3-3omega-YFP-TAP2 under the control of a 35S promoter (Chang et al. 2009), or immunoprecipitation studies using 14-3-3 antibodies (Paul et al. 2009). The total number of proteins identified in this way is close to 750. The challenge now, is to sort out which proteins are contaminants (i.e., bind nonspecifically), which proteins can be classified as secondary targets (i.e., they bind to 14-3-3s, but indirectly as part of a 14-3-3/target complex) and finally which proteins are the primary targets (i.e., they physically bind to the 14-3-3 proteins (the gold standards)). For mammalian high-throughput data, Johnson et al. recently considered criteria that may help to preselect interesting candidate proteins (Johnson et al. 2011). They visualized sixteen 14-3-3 binding studies with more than 1,800 proteins using VisANT (http//:visant.bu.edu) and applied it in such a way that proteins identified most frequently moved to the center of the display. Proteins in the center of the graph were selected for further experimental evaluation. One interesting outcome of this exercise was that of the 152 gold standard 14-3-3 targets only half of these were discovered in the 16 high-throughput studies (Johnson et al. 2011). Figure S1 shows the analysis of overlap in plant 14-3-3 interactomics studies using the VisANT program (http//:visant.bu.edu). All green dots represent unique target proteins and even in the study where the same plant extract was used in combination with three 14-3-3 isoforms (Shin et al. 2011) most proteins identified are isoform specific. However, a number of targets were identified in three or four of the pull-down studies (purple and red dots) and these targets are interesting candidates for further validation studies.

Another way to analyze these large-scale interactomics studies is to see whether proteins that function in a network or specific pathway are overrepresented. Namely, animal studies show that 14-3-3 proteins operate in networks/pathways that define a specific biological process, like apoptosis, cell cycle, etc. (Freeman and Morrison 2011; Gardino and Yaffe 2011; Hermeking and Benzinger 2006; Kleppe et al. 2011). One pathway represented by multiple putative targets in the interactomics list is glycolysis, and a recent metabolomics study corroborates the suggestion that 14-3-3 proteins have an important function in glycolysis (Diaz et al. 2011). As we will discuss below for glycolysis, it must be kept in mind that identified proteins with a function in a specific pathway may have been isolated as part of a large complex wherein 14-3-3 interacts with a single protein.

14-3-3 and glycolysis

Already in the early days of plant 14-3-3 research, it was recognized that 14-3-3 proteins are involved in the regulation of enzymes with a role in primary metabolism (Cotelle et al. 2000; Huber et al. 2002; Sehnke and Ferl 1996). The interaction with and the effect on a number of enzymes has been well described: nitrate reductase, sucrose phosphate synthase, glutamine synthetase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (for reviews, see Comparot et al. 2003; Denison et al. 2011). A first indication that 14-3-3s have a wider role in plant metabolism came from a study with sugar-starved *Arabidopsis* cells (Cotelle et al. 2000). Sugar signaling is an essential part of every plant life, as the supply of sugars changes drastically during the course of a day or in processes like germination, flowering, senescence, etc. Sugar starvation resulted in a strong increase in proteolytic cleavage of many proteins due to the loss of interaction between 14-3-3s and many targets. Recently, a large-scale metabolomics/enzyme activity study of *Arabidopsis* 14-3-3 knock-out or over-expression lines further indicated a central function of 14-3-3 proteins in glycolysis and the tricarboxylic acid (TCA) cycle (Diaz et al. 2011). 14-3-3 Over-expression resulted in lower levels of sugars and nitrogen containing compounds, what corresponded with reduced activities of known 14-3-3 targets. In line with reduced

activities of two key enzymes of the TCA cycle, isocitrate dehydrogenase and malate dehydrogenase, also the malate and citrate levels were reduced in the 14-3-3 over-expressors (Diaz et al. 2011). Remarkably, a comparison of the textbook glycolytic and TCA cycle enzymes with proteins identified in 14-3-3 affinity studies shows that enzymes in these pathways are highly represented in independent studies (Chang et al. 2009; Paul et al. 2009; Shin et al. 2011; Swatek et al. 2011; Fig. S2).

In view of efficient channeling of substrates and products, it is known that enzymes are located close to one another (Anderson and Carol 2005) or present in large enzyme complexes (Brandina et al. 2006). In the latter study in yeast, it was shown that enolase takes part in large macromolecular complexes containing additional glycolytic enzymes, mitochondrial membrane carriers, and enzymes of the TCA cycle (Brandina et al. 2006). The enolase complex may have a role in channeling pyruvate towards the TCA cycle in mitochondria. In *Arabidopsis thaliana*, enzymes of glycolysis are present free in the cytosol and on the surface of mitochondria, whereat the extent of mitochondrial association is dependent on the respiration rate (Graham et al. 2007). In the latter study, the identity of mitochondrial associated glycolytic proteins in a complex with a native molecular mass of around 350 kD was established and seven out of eight of these proteins (same At numbers) were also identified in the 14-3-3 affinity studies (Fig. S2b). Anchoring of these complexes to the mitochondrial membrane may occur through interaction of glyceraldehyde-3-phosphate dehydrogenase or hexokinase with the outer mitochondrial membrane protein, VDAC (Holtgrawe et al. 2005; Wilson 2003). VDAC3 was also identified in the 14-3-3 affinity studies (Shin et al. 2011). So, it is possible that 14-3-3 proteins bind to glycolytic or TCA cycle modules of protein complexes, where at least one protein in the complex is a direct 14-3-3 targets. A yeast two-hybrid assay is a suitable first approach to test for primary 14-3-3 targets, as shown recently for isocitrate dehydrogenase and two malate dehydrogenase enzymes (Diaz et al. 2011).

14-3-3 and protein kinases

14-3-3 Proteins have an intricate relationship with protein kinases. Besides phosphorylation of the 14-3-3 interaction site in 14-3-3 targets, (1) 14-3-3s are phosphorylated by kinases (see above), (2) kinases themselves are 14-3-3 targets, and (3) some 14-3-3 interacting kinases phosphorylate other 14-3-3 target proteins. The latter resulted in a "*piggy-back ride*" model, where the kinase delivers the 14-3-3 to its newly phosphorylated target (Ikeda et al. 2000). Whether this happens in vivo, remains to be studied. It should be noted that all targets of a kinase which interact with 14-3-3 proteins, are indirectly affected by these 14-3-3 proteins. Kinases reported to interact directly with 14-3-3 proteins are the following:

CPK1, a calcium dependent kinase

Transient changes in cytosolic Ca^{++} concentrations occur during growth, development and (a)biotic stress. CPKs act as calcium sensors to translate the calcium signatures. The CPK protein family comprises 34 isoforms in *Arabidopsis* divided in 12 subfamilies (Harper et al. 2004). CPK protein substrates function in ion and water transport, transcription, defense, and hormone signaling. A calcium-dependent kinase (AtCPK1) was the first kinase reported to interact with 14-3-3s (Camoni et al. 1998). The activity of recombinant CPK1 was stimulated by several 14-3-3 isoforms, with 14-3-3omega being the most effective. Further evidence for CPK1/14-3-3 interaction is provided by the identification of CPK1 in the tap-tagged 14-3-3 omega pull-down experiment (Chang et al. 2009). So far, the CPK1 14-3-3 interaction site(s) has not been identified. Indirectly, 14-3-3s may affect the activity of the two Ca^{++}–ATPases that are phosphorylated by CPK1: ACA2 and ACA8 (Giacometti et al. 2012; Hwang et al. 2000).

WPK4, a SNF1-related protein kinase

Plants contain a large family of SNF1-related protein kinases that play a central role in phosphorylation cascades involved in carbon assimilation and in stress adaptation (Halford and Hey 2009). The *Arabidopsis* SnRK family is subdivided in three branches with different number of members (): SnRK1 (3), SnRK2 (10), and SnRK3 (25). Ikeda et al. identified a wheat SnRK3 kinase, called WPK4, that interacts with two wheat 14-3-3 proteins (Ikeda et al. 2000). The WPK4 gene is upregulated by light and cytokinins and downregulated by nutrients. The kinase-dead mutant (K75D), lacking phosphorylation activity, showed essentially no interaction with either of the two 14-3-3s, indicating that the interaction is auto-phosphorylation dependent (Ikeda et al. 2000). Although in many cases, mutation of a single phospho-site suffices to abolish target/14-3-3 interaction, at least two phospho-sites in the WPK4 C-terminus (S^{388}; RPASLN and S^{418}; RFISGEP) are important for 14-3-3 binding. Since the double mutant S^{388}/S^{418} still showed some interaction with 14-3-3, an additional binding site(s) in the C-terminus of WPK4 was postulated (Ikeda et al. 2000). An interesting twist in this story is that the closest *Arabidopsis* homologues (CIPK12, 19, 18, and 23) do not interact with 14-3-3s, even though the two WPK4 14-3-3 interaction sites are conserved (Nozawa et al. 2003). The alignment between WPK4 and the *Arabidopsis* CIPKs shows that a proline-rich stretch N-terminal of the first 14-

3-3 interaction motif is notably absent in all AtCIPKs (Fig. S3). In barley, a homologue of the wheat WPK4 was identified in a yeast two-hybrid screen as 14-3-3 interactor (Schoonheim et al. 2007a) and this protein indeed does contain the proline-rich stretch, like all other homologues in monocot species. The proline-rich domain is predicted to be recognized by the SH3 domain present in proteins involved in vesicle trafficking and cytoskeletal elements (Lam et al. 2001) and might be a distinguishing feature between monocot and dicot SnRK3s. WPK4 is one of the kinases that has a dual 14-3-3 connection, since it also phosphorylates the 14-3-3 interaction site (S^{534}) in nitrate reductase (Ikeda et al. 2000). This is a more common theme in animal 14-3-3 biology, e.g., cell cycle checkpoint (CHK1) kinases, WEE1 kinase (Hermeking and Benzinger 2006).

MAP kinases and programmed cell death

MAPK cascades are key signaling modules for responding to extracellular (a)biotic stimuli and generating programmed developmental processes in plants (Rodriguez et al. 2010). In animal cells, MAP-kinases are well-known 14-3-3 targets (Fritz et al. 2006) and it is clear now that in plants MAP-kinases are targeted by 14-3-3s as well. The first MAP kinase shown to interact with a 14-3-3 protein (GF14-6) was the maize ZmMPK6 protein; the interaction was dependent on auto-phosphorylation but essential phosphosite(s) were not reported (Lalle et al. 2005).

In tomato, the TFT7 14-3-3 protein has a central role in MAP kinase cascades that respond to infection by *Pseudomonas syringae* pv (Oh et al. 2010). Recognition of the bacterial effectors AvrPto or AvrPtoB by Pto, a Ser-Thr protein kinase, activates the cascade and results in programmed cell death (PCD). TFT7 interacts with phosphorylated MAPKKKα (at S^{535}), what enhances the accumulation of MAPKKKα, possibly by preventing degradation of the kinase during PCD. As a result, two MAP-kinases acting downstream of MAPKKKα were found to be more phosphorylated (Oh et al. 2010). Binding of TFT7 to MAPKKKα is a critical step controlling PCD-enhancing activity. Co-expression of TFT7 and the mutated MAPKKKα (S^{535}A) suggested that there may be a second 14-3-3 interaction site in the MAPKKKα protein. As mentioned above, MAP kinases act in a cascade and recent evidence shows that 14-3-3 proteins go along with the phosphorylation cascade. Namely, the MAP-kinase acting downstream of MAPKKKα, MKK2, interacts with 14-3-3s as well (Oh and Martin 2011). The 14-3-3 interaction site in MKK2 is in the N-terminus of the kinase, with T^{33} as phosphorylated residue; importantly, the 14-3-3 motif is conserved in orthologues from rice, alfalfa, tobacco and *Arabidopsis*. Oh et al. suggested that the 14-3-3 dimer may act as a scaffold here, holding a kinase in the binding groove of each monomer, thus ensuring efficient transfer of the phosphorylation cascade (Oh et al. 2010).

WEE1 and cell cycle arrest

The cell cycle is an evolutionarily conserved mechanism in eukaryotic cells, from yeast to plants and animals. The cell cycle is regulated by a series of cyclin-dependent protein kinases (Cdcs) that drive cells through specific transitions, notably at G1/S and G2/M. At the G2/M transition, Cdcs are positively phosphoregulated by CDC25 phosphatase and negatively by the nuclear dual function kinase WEE1. In animal cells, 14-3-3 proteins are master regulators of the cell cycle because they interact with Cdcs, CHK1, CDC25, and WEE1 (Hermeking and Benzinger 2006).

Recent evidence shows that at least part of this 14-3-3 function in cell cycle control is preserved in plant cells. Namely, 14-3-3 proteins do interact with plant WEE1 kinase (Grønlund et al. 2009). Interaction between WEE1 and 14-3-3 omega was found in the nucleus of interphase cells; an S^{485}A mutation of WEE1 abolished this interaction in vitro and altered the in vivo spatial interaction, indicating that S^{485} is a likely regulatory phosphorylation target for the WEE1/14-3-3 omega interaction (Grønlund et al. 2009). A recent study showed that under normal growth conditions, the plant WEE1 protein is not rate limiting for cycle progression (De Schutter et al. 2007). However, when cells suffer DNA damage, WEE1 is targeted by ATR–ATM signaling kinases and cell cycle arrest is induced. *wee1* mutant plants are hypersensitive to replication blocking agents, such as hydroxyurea or aphidicolin (De Schutter et al. 2007). The role of 14-3-3 in WEE1 function can be addressed by complementation of the *wee1* mutant plants with the mutated WEE1 gene (S^{485}A).

NIMA-like kinases, SPAK and PNEK1

The first never in mitosis A (NIMA)-like kinase was isolated during a mutant screen in the filamentous fungus *Aspergilus nidulans* (Oakley and Morris 1983). The mutants were found to be never in mitosis and the gene corresponding to this phenotype was named NIMA. Since then, NIMA-like genes (NEKs) have been found in all eukaryotic organisms and their main function is in mitotic processes like mitotic entry, spindle formation, cytokinesis, etc. (O'Connell et al. 2003). The first plant Nek was identified in tomato during a cDNA library yeast two-hybrid screen using the SELF PRUNING (SP) gene as bait (Pnueli et al. 2001). The SP gene is the tomato ortholog of CENTRORADIALIS and TERMINAL FLOWER1, genes which maintain the indeterminate state of inflorescence meristems in *Antirrhinum* and *Arabidopsis*, respectively (Pnueli et al. 1998). The screen with SP resulted in a number of SP interacting proteins (SIPs), among these were the NEK protein SPAK, a bZIP transcription factor (SPGB) and two 14-3-3

proteins. Intriguingly, further analysis showed that the SIPs form a network of bipartite interactions (see also below, flowering). Here, we will focus on the 14-3-3/SPAK interaction. One 14-3-3 interaction motif was identified in SPAK and mutational analysis of $S^{406}A$ showed that this serine is indispensible for 14-3-3 interaction (Pnueli et al. 2001). The S^{406} interaction site is conserved in the poplar SPAK homologue, PNek1, that was identified in a yeast two-hybrid cDNA screen using 14-3-3 as bait (Cloutier et al. 2005). These authors confirmed the importance of S^{406} for 14-3-3 interaction and in addition provided evidence that also the PNek1 C-terminal coiled-coil dimerization domain is important for 14-3-3 interaction. Intriguingly, the $S^{406}A$ mutated SPAK protein not only lost interaction with 14-3-3, but also with the SP protein (Pnueli et al. 2001). This suggests that SP and 14-3-3 compete for the same S^{406} motif in SPAK for binding. A question unanswered thus far is what the consequence of 14-3-3 binding is for SPAK and PNek1 functioning. Does it affect the kinase activity, block interaction with SP, alter subcellular localization, stability, etc.? SPAK antisense tomato plants have a clear elongated fruit phenotype (Pnueli et al. 2001) and complementation of *spak* mutant plants with a $SPAK^{S406A}$ mutant gene may give insight into the role of 14-3-3/SPAK interaction in plant development.

What about the possibility that 14-3-3 proteins play a role in the function of other Nek-kinases? In *Arabidopsis*, seven *Nek* genes have been identified (Vigneault et al. 2007) and the most important 14-3-3 interaction site around S^{406} is conserved in Nek1-4, but is absent in Nek5-7 (Table 2). AtNek2 has a critical role in plant survival and is involved in the control of cell morphogenesis and plant development, most probably through an interaction with microtubules (Agueci et al. 2012). *AtNek2*, RNAi mutant seedlings are delayed in development, bolting and the emergence of the inflorescence are slowed, leaves were smaller and thinner, and normal epidermal cells were substantially larger in RNAi leaves as compared to wild type (Agueci et al. 2012). Based on these phenotypes, it will be very interesting whether 14-3-3/AtNek2 interaction plays a role in AtNek2 function.

14-3-3 and bZIP-transcription factors: players in flowering and hormonal signaling

One of the first articles on plant 14-3-3 proteins reports that 14-3-3 proteins are part of a complex that binds to a cis-acting promoter element, called G-box (hence the name G-box binding protein; De Vetten and Ferl 1994). Soon, other components of this complex were identified as the basic leucine zipper transcription factor EmBP1 and the transcriptional activator VP1 (Schultz et al. 1998). EmBP1 belongs to the group G bZIP transcription factors (Jakoby et al. 2002) and now different classes of these bZIPs have been implicated as targets for 14-3-3 proteins affecting different biological processes. We will describe how 14-3-3 interaction affects the different bZIP Groups and how this influences flowering, abscisic acid (ABA) and gibberellic acid (GA) signaling.

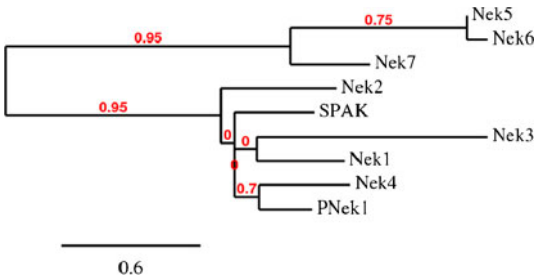
14-3-3 Interaction with group A bZIP

FD and the floral initiation pathway

Our understanding of how flower induction is controlled in plants has made enormous progress in recent years, as reviewed recently (Amasino and Michaels 2010; Wigge 2011; Zeevaart 2008). Four interacting flowering pathways can be distinguished: the cell autonomous, gibberellin, the vernalization, and the photoperiodic pathways. We will focus on the latter since the role of 14-3-3 proteins herein takes shape. The photoperiodic pathway accelerates flowering under inductive conditions, like long days in *Arabidopsis* for example. Heart of this day-length measurement system in the leaves, leading to flower induction, is the circadian regulation of CONSTANS (CO) expression and subsequent photoperiodic expression of the Flowering Locus T (FT) gene. The FT protein is made in the phloem companion cells and it is now generally agreed upon that it is the FT protein that is the graft transmissible signal (*the florigen*) that travels in the phloem towards the shoot apex

Table 2 Conservation of the two SPAK 14-3-3 interaction sites (S274 and S406) in PNek1 and seven *Arabidopsis* Nek homologues

	S274	S406
SPAK	LNGP RRN S LP AC	VSTT RRA S LP LT
PNek1	MNSP RQN T LP FQ	NPTT RRT S LP LP
Nek1	LNNL RRK T LP PE	IPSA RRT S LP LT
Nek2	VNDPGSNVLPAQ	AALI RRA S MPSS
Nek3	LSF REHD T LP SE	AVVT RRA S LP IS
Nek4	LESP RRS T FP LQ	TERR RRV S LP LV
Nek7	LSPIYLPVFPIK	ETPAEENALPKE
Nek5	LSPVFKPVVSKS	TAIWLTKSLMNV
Nek6	LSPVFKPVVDKS	TAIWLTKSLMNV



The importance of the S274 site is not as clear as that of S406 (Cloutier et al. 2005). Based on the conservation of these 14-3-3 motifs, Nek1, 2, 3, and 4 are likely candidates as 14-3-3 target proteins; the putative 14-3-3 interaction sites are marked in yellow and the putative phosphor-Ser/Thr in green

(Wigge 2011; Zeevaart 2008). According to recent reviews, FT interacts in the shoot apex with FLOWERING LOCUS D (FD), a group A bZIP transcription factor and this promotes transition to flowering by activating two genes: SUPPRESSION OF OVEREXPRESSION OF CO1 and APETALA1 (Amasino and Michaels 2010; Wigge 2011). However, this model is incomplete, since it is clear now that 14-3-3 proteins play an intriguing and complex role in the interaction between FT and FD.

Yeast two-hybrid assays showed that 14-3-3 proteins interact with FT, Hd3a (rice orthologue of FT), SFT (tomato orthologue of FT) and also with the floral repressors Brother of FT (TFL1) and SP (Lifschitz et al. 2006; Pnueli et al. 2001; Purwestri et al. 2009). Yeast two-hybrid assays also point to a direct interaction between FT and FD and a phospho-serine in the FD C-terminus was identified as being essential for FT/FD interaction (Abe et al. 2005). Also, the tomato FD orthologue, SPGB, was reported to interact with the tomato SP protein, the *Arabidopsis* FT protein, and the tomato SFT protein (Lifschitz et al. 2006). In spite of all this evidence, a recent study with the rice Hd3a protein questions the model wherein the FT and FD protein directly interact, because pulldown experiments with purified Hd3a, OsFD1 and GF14c (rice 14-3-3 protein) showed interaction between Hd3a↔14-3-3, OsFD1↔14-3-3 but not between OsFD1 and Hd3a (Taoka et al. 2011). And since OsFD1 combined with GF14c did pulldown Hd3a, the authors conclude that 14-3-3 proteins form a "bridge" between FT and FD: OsFD1↔GF14c↔Hd3a, thus forming the *florigen activation complex* (FAC; Taoka et al. 2011). Unfortunately, the crystal structure of GF14c, Hd3a and a phospho-peptide derived from the last nine amino acids of OsFD1 does not provide clear evidence for this "bridge model" (Taoka et al. 2011). In the crystal structure, OsFD1 binds in the 14-3-3 binding groove by means of a canonical 14-3-3 interaction motif (S^{192} phosphorylated) and Hd3a abuts the C-terminal region of GF14c. Since only a short peptide of OsFD1 was co-crystallized, it remains a question whether and how OsFD1 binding to 14-3-3 stabilizes the interaction between Hd3a and 14-3-3.

These recent studies on 14-3-3 and flowering bring a new model with a role for 14-3-3 proteins at three key positions in the photoperiodic pathway (Fig. 1):

1. CONSTANS, the central element that coordinates light and clock inputs to trigger FT expression, interacts with 14-3-3 proteins (Mayfield et al. 2007)
2. in the phloem companion cells, 14-3-3 interaction with FT (demonstrated for Hd3a and GF14c in rice) prevents "leakage" of FT into the phloem elements through the plasmodesmata, thus ensuring FT accumulation over time (the "trapping model", (Purwestri et al. 2009))
3. in the shoot apex, where floral meristem identity genes are upregulated, FT combines with 14-3-3 and FD to form the florigen activation complex and initiate flowering (Taoka et al. 2011)

This model, which combines data from *Arabidopsis*, rice, and tomato does leave or raise a number of questions. First, the role of 14-3-3 proteins in the functioning of CO is not clear yet. Secondly, the trapping model is in line with the conclusion that the Hd3a/GF14c interaction acts as *negative* regulator of flowering (Purwestri et al. 2009). But, in the apex 14-3-3 acts as a *positive* regulator of flowering as central component of the FAC. These opposite functions of 14-3-3 may be the result of temporal or spatial differences in 14-3-3 isoform expression. Another important question is whether in the companion cells of leaves a trimeric complex is formed as suggested as the case in the apex. If so, then it is

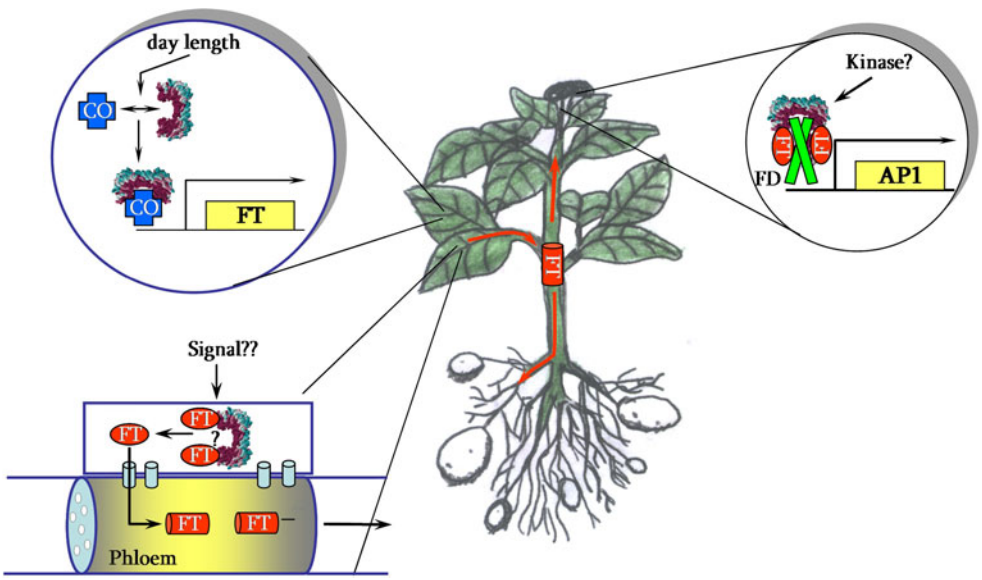


Fig. 1 Model showing the role of 14-3-3 in the flowering process through interaction with CONSTANS in the leaves, interaction with *FT* in the phloem companion cells and the formation of the *florigen activation complex* (FAC) in the shoot apex with FD and FT. It remains to be seen whether 14-3-3 can form a trimeric complex with other bZIP transcription factors and FT or related proteins like TFL1. Also the role of these complexes in tuberization of, e.g., potatoes remains to be studied

unlikely that FD is involved due to its tightly controlled expression in the shoot apex (Abe et al. 2005). However, other members of the group A bZIP transcription factors (ABF/ABI5/AREB family), are well known (gold standard) 14-3-3 targets with a 14-3-3 binding motif at the very C-terminus comparable to FD (Schoonheim et al. 2007b; Sirichandra et al. 2010). As summarized in Table S3, there are at least two other bZIPs with the same LxRxx(S/T)AP 14-3-3 binding motif as present in FD at the extreme C-terminus. We therefore postulate that other bZIP proteins, many of which are stress responsive, may take the place of FD in the trimeric complex in organs other than the shoot apex. These bZIPs may combine with FT, or related proteins like TFL1 (brother of FT) to control flowering under high salt stress (Ryu et al. 2011) or control the transition to tuberization in potato in dependence of day length (Navarro et al. 2011). Another question, not addressed so far, is what "signal" induces the dissociation of the FT/14-3-3/bZIP complex in phloem companion cells and starts the flow of "florigen" to the apex.

ABF/ABI5/AREB and ABA signaling

Other members of the group A bZIP proteins bind to a so-called ABA-responsive element, a promoter element similar to the G-box sequence and found in almost all ABA responsive genes. These bZIP proteins are known as the ABF/AREB/ABI5 family (Kim 2006). Four members of this group in barley were shown to interact with 14-3-3 proteins and this interaction was essential for the ABA induced transactivation activity of HvABI5 (Schoonheim et al. 2007b). A canonical 14-3-3 interaction site was identified and verified to be present at the very C-terminal end of all four barley bZIPs (conserved in their *Arabidopsis* homologues). Intriguingly, the position and structure of the 14-3-3 interaction motif is very similar to those present in FD and related transcription factors (Table S3; Fig. 2). Recently, it was elucidated how ABA drives the interaction between 14-3-3 and AtABF3 and how this interaction positively affects the AtABF3 trans-activation activity (Sirichandra et al. 2010). These authors first showed that the motif around T^{451} in the ABF3 protein forms a phosphorylation motif of the SnRK2.6 kinase OST1. OST1 is activated by ABA, because binding of ABA to its receptor inhibits the phosphatases ABI1/2 that keep OST1 dephosphorylated under nonstress conditions. Next, they showed that OST1 interacts with ABF3 in the nucleus and phosphorylates T^{451}. Binding of 14-3-3 to the phosphorylated ABF3 results in inhibition of proteasomal break down, an increase in ABF3 concentration, and activation of ABA-inducible genes (Sirichandra et al. 2010). So, the 14-3-3/ABF3 interaction can be classified as a "*protein stabilization*" effect reported more often as 14-3-3 mechanism (Cotelle et al. 2000; Lee and Lu 2011). Complementation of the *abf3* mutant plant with the ABF-T^{451}A mutant gene/protein failed, because 14-3-3 could not stabilize the ABF3 mutant protein. These results are in line with the 14-3-3RNAi experiments in barley aleurone protoplasts, that showed that silencing of specific 14-3-3 isoforms suppressed the ABA induction of ABRC3-GUS reporter gene (Schoonheim et al. 2007b).

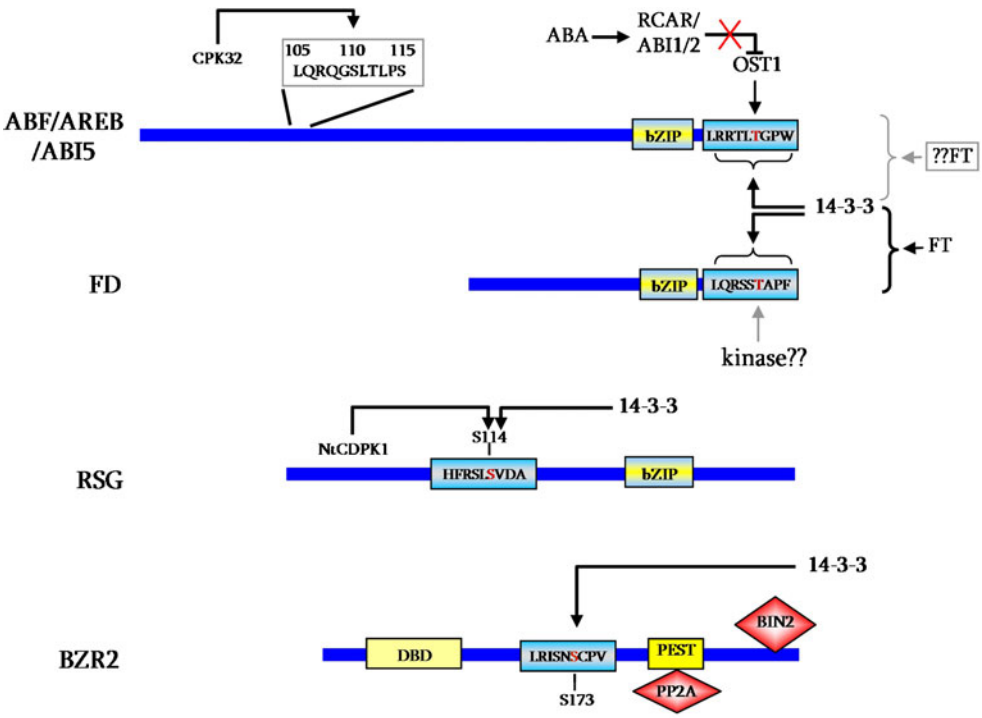


Fig. 2 Structure of different (bZIP)-trancscription factors (ABF/AREB.ABI5, FD, RSG, and BZR2) and the location of the identified site of 14-3-3 interaction

14-3-3 Interaction with class I bZIP

RSG and GA signaling

REPRESSION OF SHOOT GROWTH (RSG) is a group I bZIP transcription factor, identified in tobacco as a regulator of endogenous GA levels (Fukazawa et al. 2000). RSG binds to and activates the promoter of the ent-kaurene-oxidase (KO) gene, encoding an important enzyme in the GA biosynthesis pathway. More recently, it was shown that RSG also activates the NtGA20ox1 gene through direct binding to its promoter where a negative feedback loop controls GA controlled GA biosynthesis with a central role for 14-3-3 proteins (Fukazawa et al. 2010). 14-3-3 Proteins were shown to bind to the tobacco RSG protein through the motif around phospho-S^{114} (Igarashi et al. 2001; Ishida et al. 2004) and the S^{114} phosphorylating kinase was identified as NtCDPK1 (Fig. 2; Ishida et al. 2008). The closest *Arabidopsis* homologue of NtCDPK1 is CPK21, a calcium-dependent kinase involved a.o. in stomatal regulation through phosphorylation of the anion channel SLAC1 (Franz et al. 2011). In this pathway, a very interesting feedback loop was identified: RSG activates transcription of GA biosynthesis genes (KO, GA3ox and GA20ox) what results in an increase in intracellular GA, cell elongation, and growth. GA also stimulates the activity of NtCDPK1, what results in enhanced RSG-S^{114} phosphorylation. Subsequently, phosphorylated RSG interacts with 14-3-3 proteins and the RSG/14-3-3 migrates *out of* the nucleus, resulting in reduced GA biosynthesis.

Group I of the *Arabidopsis* bZIPs harbors three proteins that show homology with RSG and have the conserved 14-3-3 interaction motif around S^{114}. One of these homologues is the VirE2-interacting protein 1 (VIP1), required for VirE2 nuclear import and *Agrobacterium* tumorigenicity (Tzfira et al. 2001). Clearly, facilitating the nuclear import of the virulence protein VirE2 of *Agrobacterium* is not the "normal" function of VIP1. A number of seemingly unrelated functions have been ascribed to VIP1: VIP1 has functions in plant immunity signaling (Pitzschke et al. 2009), low sulfur tolerance (Wu et al. 2010), and acts as regulator of osmo-sensory signaling (Tsugama et al. 2012). Tsugama et al. show that, whereas RSG controls expression of GA biosynthesis genes, VIP1 enhances the promoter activities of *CYP707A1/3*, two genes encoding proteins with ABA 8′-hydroxylase activity, involved in ABA catabolism. During drought ABA accumulates and after rehydration (when turgor pressure goes up) VIP1 migrates within 10 min to the nucleus and activates *CYP707A1/3* gene expression. In analogy with the model for RSG/14-3-3 interaction, binding of 14-3-3 to VIP1 may alter the amount of nuclear VIP1 by either affecting nuclear import

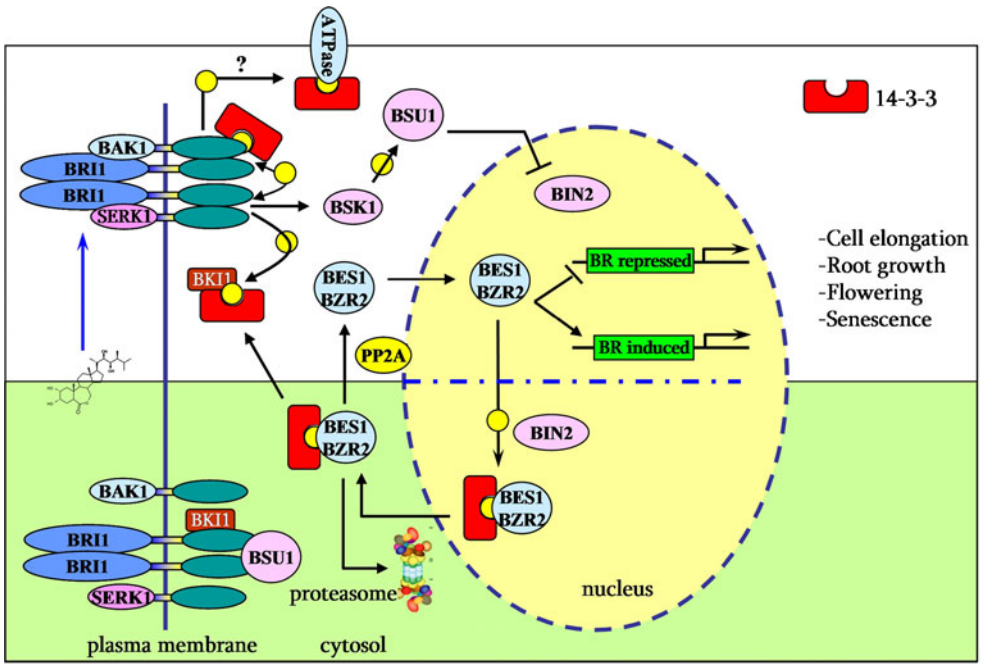


Fig. 3 The role of 14-3-3 proteins in BR signaling. At low/no BR, BKI1 interacts with and inhibits BRI1. BIN2 phosphorylates BES1/BZR2, 14-3-3s bind and mediate nuclear export. With BR, BRI1 is activated, phosphorylates BKI1, resulting in membrane release of BKI1 and association of the different co-receptors with BRI1. BRI1 phosphorylates the kinase BSK1, which phosphorylates the phosphatase BSU1. BSU1 then represses BIN2 activity and stimulates proteosomal breakdown. BKI1 competes with BES1/BZR1 for bound 14-3-3s, BES1/BZR1 are dephosphorylated by the phosphatase PP2A and shuttle to the nucleus. Here, BES1/BZR1 bind to specific promoter elements (with other factors) and either repress or stimulate the expression of genes with a function in the ultimate BR responses at the cell and whole plant level

through interference with the putative NLS in the N-terminus, or affect nuclear export through a putative NES in the C-terminus of the protein (Tsugama et al. 2012). Activation of *CYP707A1/3 expression is* also highly relevant for controlling stomatal closure, to ensure ABA catabolism during an increase in external humidity (Okamoto et al. 2009).

14-3-3 and bHLH transcription factors BZR1/BES1

14-3-3 Proteins are functionally involved in the brassinosteroid (BR) pathway through their interaction with two basic helix-loop-helix-like transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1/BZR2) and BRASSINAZOLE-RESISTANT 1 (BZR1; Bai et al. 2007; Gampala et al. 2007). BES1 and BZR1 share 88 % identity and they have both redundant and distinctive functions, as evidenced from the analysis of their numerous target genes (Sun et al. 2010; Yu et al. 2011). BES1 and BZR1 coordinate BR homeostasis and signaling as they play a dual role in both positive (induction of their own expression) and negative (inhibition of many genes in BR biosynthesis and signaling) feedback loops (He et al. 2005; Sun et al. 2010; Yu et al. 2011).

Multiple *Arabidopsis* 14-3-3 proteins (14-3-3 lambda, kappa, epsilon, phi, and omega) were identified as BZR1/BES1-interacting proteins in yeast-two hybrid screens for BZR1/BES1-interacting proteins (Gampala et al. 2007; Ryu et al. 2007). The 14-3-3 interaction site in BZR1 and BES1 was shown to be around the phosphorylated S^{173} and S^{171} residue, respectively (Gampala et al. 2007; Ryu et al. 2007) and is conserved across species, as shown for the rice homologue OsBZR1 (Bai et al. 2007). Nuclear localized BRASSINOSTEROID-INSENSITIVE 2 (BIN2) kinase has a predominant function in the phosphorylation of the BES1 14-3-3 motif and formation of a BES1/14-3-3 complex results in enhanced nuclear export. BIN2 phosphorylation of BES1/BZR1 is balanced by the phosphatase PP2A and analysis of several PP2A subunit mutants showed that PP2A activity is required for normal BZR1 dephosphorylation and plant growth (Di Rubbo et al. 2011; Tang et al. 2011). PP2A has a dual role in the BR pathway since it is also involved in the termination of BR signaling through dephosphorylation of the BRASSINOSTEROID-INSENSITIVE1 (BRI1) receptor what promotes BRI1 degradation (Wu et al. 2011). Evidence is accumulating that 14-3-3 proteins have a role in the plasma membrane localized BR-receptor complex too. First, the receptor kinases SERK1

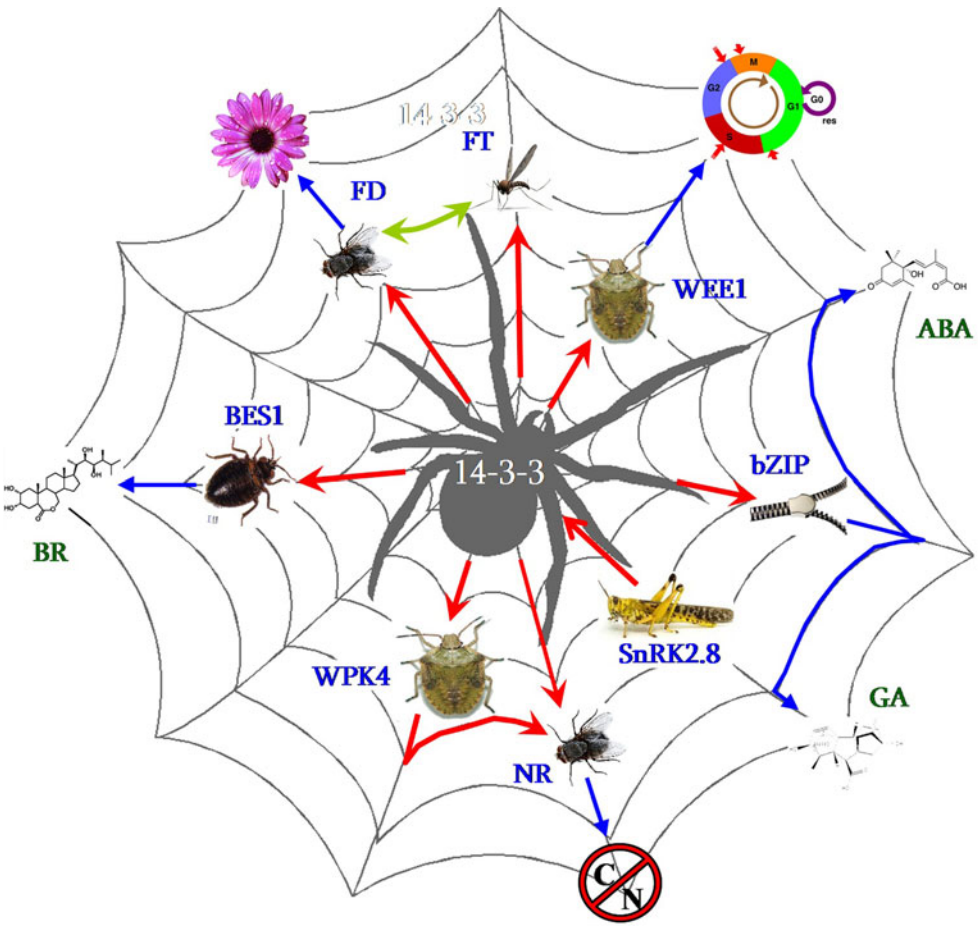


Fig. 4 14-3-3 Proteins as spiders in the phospho web. Interaction with a wide range of phosphorylated target proteins affects different cellular processes, like flowering, cell cycle, ABA, GA, and BR synthesis/signaling and carbon/nitrogen metabolism

and BAK1 that form a complex with BRI1 (Karlova et al. 2006), were reported to be in a complex with 14-3-3 proteins (Karlova et al. 2006; Rienties et al. 2005), or to interact directly in a yeast two-hybrid assay (Schoonheim et al. 2007a). Secondly, a negative regulator of BRI, called BKI1 (BRI1 KINASE INHIBITOR 1), that keeps the receptor in a quiescent state when there is no BR, was shown to interact with 14-3-3s and affect BR-signaling in a dual fashion (Wang et al. 2011). At increasing BR concentrations, hormone-bound BRI1 phosphorylates BKI1 on residues S^{270} and S^{274}. Phosphorylation of BKI1 stimulates the release of BKI1 from the plasma membrane and now positive regulators like BAK1 and SERK1 can interact with BRI1 and activate BR signaling (Wang et al. 2011). Mutational analysis showed that BKI1 phosphorylated at $S^{270}/^{274}$ gains affinity for 14-3-3 proteins and the BKI1 interaction with 14-3-3 has two consequences: (1) it prevents BKI1 from being dephosphorylated and return to the plasma membrane and (2) it activates the downstream BES1/BZR1 transcription factors through competing for the 14-3-3 proteins bound to BES1/BZR1 in the cytosol. So, after its release from the plasma membrane, BKI1 acts as a positive regulator of BR-signaling (Fig. 3). It will be important to study how exactly BKI1 interacts with 14-3-3 proteins because the interaction motif is rather unusual as it involves two phosphorylated serines and there is no R or K at the −3 or −4 position. Then there are two more 14-3-3 related events that need further study, namely the phosphorylation of 14-3-3lambda by SERK1 (Rienties et al. 2005) and the BRI1 interaction with P-type H^+-ATPases in the plasma membrane (Caesar et al. 2011). BL-induced cell wall expansion (necessary for cell elongation) is accompanied by upregulation of P-ATPase activity. FRET analysis showed that BRI1 associates with AHA1 in *Nicotiana* protoplasts and one hypothesis to explain the increase in pump activity is that BRI1 phosphorylates the penultimate Thr of the P-ATpases thus creating affinity for 14-3-3 binding and releasing the C-terminal imposed autoinhibition (Caesar et al. 2011).

Summary and perspective

Compared with the number of gold standard 14-3-3 targets in animals, the number of gold standards in plants is still relatively limited. However, many putative targets have been identified and validation of more targets as gold standards will be invaluable to understand the cellular fine-tuning mechanisms that underlie plant growth and development. Although 14-3-3 proteins are isoform specific phospho-proteins in their own right, we know nothing yet about how phosphorylation affects plant 14-3-3 function and which kinases are involved. Kinases themselves are emerging as a major category of 14-3-3 targets and since kinases are strong amplifiers of internal or external signals affecting multiple proteins, it is clear that the effects of 14-3-3 are far reaching. To control the complete "phospho-balancing mechanism", 14-3-3 proteins are expected to interact with phosphatases as well, but none has been identified yet. Nevertheless, the interactomics studies have identified many phosphatases or their subunits as putative targets that await further validation.

The similarity in sequence and position of the 14-3-3 binding site in the bZIP transcription factors, raises the question whether the same or different kinases are involved in phosphorylation of the binding motif and also whether FT and related proteins can form a trimeric complex with all bZIPs that share this C-terminal 14-3-3 interaction site.

In view of the omnipresence of phosphorylation in 14-3-3 function, 14-3-3 proteins can be considered as spiders in a web of phosphorylation related events (Fig. 4). An important goal for the near future is the identification of the kinases that phosphorylate target 14-3-3 sites, since this will provide insight in the upstream signaling pathways.

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