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Hormonal regulation of tolerance to cold stress of *Arabis alpina* from the French Alps and Rila mountain

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USED ABBREVIATIONS

AAO – abscisic acid oxidase
ABA – abscisic acid
CK – cytokinins
CKX – cytokinin oxidase/dehydrogenase
cZ – cis-zeatin
cZR – cis-zeatin riboside
cZRMP – cis-zeatin riboside monophosphate
DHZ – dihydrozeatin
DHZMP – dihydrozeatin riboside monophosphate
DHZR – dihydrozeatin riboside
DPA – dihydrophaseic acid
iP – isopentenyladenine
iPR – isopentenyladenine riboside
iPRMP – isopentenyladenine riboside monophosphate
IPT – isopentenyl transferase
LHCII – light harvesting complex II
MR – modulated 820 nm light reflection
NCED – 9-cis-epoxycarotenoid dioxygenase
NT – non-tolerant plants population
P680 – reaction centre of PS II
P700 – reaction centre of PS I
PA – phaseic acid
PF – prompt fluorescence
PQ – plastoquinone
PQH_2 – plastoquinone in the reduced form (plastoquinol)
PS I – photosystem I
PS II – photosystem II
Q_A – the primary quinone acceptor in PS II
Rubisco – ribulosobisphosphate carboxylase/oxygenase
ROS – reactive oxygen species
T – tolerant plants population
tZ – trans-zeatin
tZR– trans-zeatin riboside
tZRMP – trans-zeatin riboside monophosphate

01. INTRODUCTION

As sessile organisms, plants are a subject to constant changes in the parameters of the environment: from daily and seasonal cyclic variances to sudden climatic fluctuations. Therefore, plants have evolved a vast composition of mechanisms to cope with the unfavourable conditions of the environment, in which they live. In the last years, a substantial progress in relation to our knowledge about the tolerance to conditions of low temperature in plants has been achieved. Regardless of the global warming of earth climate, the tolerance to cold temperatures is of particular interest, because early warm periods in winter are observed more and more frequently, and that leads to interruption in winter dormancy of the agriculturally significant crops, damaging them after reversal of the low temperatures (Beniston, 2005; Eccel et al., 2009).

Cold stress exerts complex effect on plants. It damages mainly the cell membranes and proteins, resulting in large amount of disadvantageous effects on the plant cell and the whole plant organism, as well (Nishida and Murata, 1996). Conditions of low positive temperatures cause the growth of plants to stop, the photosynthesis decreases, the roots stop absorbing water and mineral nutrients, and, as a consequence leaves lose their turgor and chlorosis is induced. Prolonged exposure to low temperatures and treatment with temperatures below zero induce necrotic spots on the leaf surface, due to damage of the membranes, formation of reactive oxygen species (ROS) and oxidation of structures and components of the cell. The main effect of the low temperatures is dehydration, caused by disruption of membrane functions and damage to membrane-associated proteins. The selective permeability of the cell is destroyed, so that the cell starts losing water and the roots are not able to restore the water balance. Plant cells lose their turgor and the plant wilts. Besides that, photosynthesis cannot function properly when plants are exposed to light in combination with low temperatures. As a result, reactive oxygen species (ROS) form, additionally damaging the proteins, DNA and oxidizing the membrane lipids. A greater disruption of membrane permeability causes vacuole content to leak out (main part of which are the organic acids) and cell structure is damaged.

Depending on the temperatures plants are exposed to, cold stress can be differentiated in two types: when temperatures are above zero (form 15 ^{o}C to 0^{o}C, it is called *chilling* stress) and below zero (*freezing* stress). Except the deteriorating effect of low temperatures upon cell components, when temperatures are below zero, additional danger from formation of ice crystals appears, as well as from mechanical disruption of the cell.

Cold stress exerts the greatest injuries on the process of photosynthesis. This was the reason to dedicate a big part of the current work on photosynthesis, photosynthetic activity, photosystem functioning in stress conditions, as well as the quantitative determination of structural components of photosynthesis, such as photosynthetic pigments and genes. Photosynthesis cannot function properly in conditions of cold temperatures combined with light and as a result reactive oxygen species (ROS) form in large amounts. Despite the antioxidant system of plants ROS damage the cell components with a high rate, when their formation in the photosynthetic apparatus could not be neutralised. Therefore, in the current work we analysed the effect of low temperatures on the plants photosynthetic apparatus and the role of ROS in realization of the damages. Being the main stress hormone and inductor of the dormancy in plants, ABA and its degradation products were studied. Mainly ABA is responsible for closing of the stomata and for plant resistance against dehydration (Beardsell and Cohen 1975). Considering that some of the effects of cold stress are also similar to the effects of dehydration, ABA accumulates in plants and regulates their functions after cold stress, as well. It is known, that ABA is also an important part from the plant acclimation to very low temperatures below zero (Mantyla et al. 1995; Gusta et al. 1996). Although the cytokinins, in contrast to ABA are hormones of plant growth and development, it appears, that they affect physiological processes in conditions of stress, as well (Hare et al., 1997; Davies et al., 2004). They are also the main regulators of the processes of ageing, as well as the photosynthesis. In *Arabis alpina*, particularly, in contrast to a large part of the plants from the Brassicaceae family, for example *Arabidopsis* cis-zeatins act as an important part of the hormonal regulation in cold stress conditions. Thus, as an addition to the analysis of the ABA content and the transcription of genes from the ABA metabolism, the study of cytokinines in the regulation of plants to cold stress draw a greater attention.

02. LITERATURE REVIEW

02.01. Common characteristics of the cold stress. Physiological and molecular levels of effect.

The definition of stress is every external factor, causing unfavourable effects upon plants. Depending on factors that cause them, two types of stress are known: biotic and abiotic. The biotic stress is caused by biologic agents, called pathogens (viroides, viruses, bacteria, fungi, herbivores, parasitic plants). The abiotic stress includes factors from the environment like high and low temperatures, drought, salinity stress (high concentration of NaCl or other salts), mineral deficit, hypoxia and anoxia (after hyperhydration of the roots, flooding). Cold stress can be two types, as well: *chilling* stress (low positive temperatures) and *freezing* stress (temperatures below 0^{o}C). Based on their way of dealing with low temperatures there are three groups of plants. Plants from the first group are able to survive the effects of low temperatures by the means of certain anatomical, physiological and biochemical characteristics. Plants from the next group can survive brief periods of exposure to cold stress. The third group of plants evade the stress periods in the form of seeds in the case of the annual plants, or underground parts (tubers, roots etc.) in the case of the perennial plants (Taiz and Zeiger, 2006).

It is well-known, that the main target of low temperatures are the biological membranes. The latter lose their liquidity and can be more easily destroyed in cold stress conditions, especially when they lack enough polyunsaturated fatty acids (Williams et al., 1988). This leads to leakage of the water content out of the cell, as well as valuable for the cell substances, together with the water. When the concentration of polyunsaturated fatty acids in the cell membranes is higher, the cell can survive at lower temperatures. Since the polyunsaturated fatty acids have lower melting point and exist in liquid state at lower temperatures, their presence inside the cell is a requirement for its higher tolerance to cold stress by retaining the elasticity of the membranes (Palta et al., 1983).

Proteins are also influenced by the low temperatures, as they lose their hydration shell and their hydrogen bonds break, so that the proteins shrink and their denaturation is eased. After sufficiently strong induction of low temperatures on the proteins of the cell, their cysteine groups bond irreversibly, forming disulphide bridges and thus they cannot restore their native form after rising of the temperature. As a consequence, the proteins cease to function normally. Being an important part of the cell membranes (with permeability as their main function), the denaturation of proteins also exerts a dehydration effect on the cell.

Besides that, the function of proteins as enzymes makes them an important part from almost every biological process and their impairment affects significantly the intensity of the life functions of the cell (Taiz and Zeiger, 2006).

Other very important aspect of the stress in conditions of low temperatures is the ice formation and damages caused by it. After temperature of plant tissues drops below 0^{o}C, the water inside them starts turning into ice, which does not occur instantly. Initially, the temperature decreases quickly few degrees below zero, without formation of ice. This process is known as supercooling. Immediately after that in the apoplastic system ice starts to form, and simultaneously temperature rises and stays around zero, since a process of releasing the latent heat occurs. After most of apoplast water has turned into ice, temperature starts to decrease again, but this time ice forms inside the cells, as well (Brown et al. 1974).

Cold stress affects membranes of the cell, mostly the thylakoids as well as proteins, thus stopping growth of plants and decreasing or completely inhibiting the photosynthetic activity. Inside cells of tolerant to cold temperatures plants, polyunsaturated fatty acids are synthesized, sustaining the stability and the liquid state of cell membranes at lower temperatures (Nishida and Murata, 1996). Besides that, low temperatures inhibit absorption of water and mineral nutrients from the roots, which in combination with the water leakage from the cells is a reason for overall dehydration of the plant.

When cold stress is exerted in the presence of enough light, plant cells suffer from photodamage as well (Takahashi and Murata, 2008). The light-harvesting complexes and photosystems are still intact then, but the fixation of CO_2 is lower because of the lower speed of the biochemical reactions from the Calvin cycle and the electron acceptor $NADP^+$ depletes. This causes electrons, donated from photosystems, instead of being transported for $NADP^+$ reduction to bind with oxygen and thus to form ROS, which damage the cell (Wise, 1995). Therefore, evergreen plants from the moderate, arctic and alpine areas decrease the amount of their light-harvesting complexes during winter. Moreover, the protein PsbS / CP22 (a peripheral protein form PS II, associated with LHCII) turning excess unused light energy in the form of heat, accumulates in higher concentration. The energy, absorbed from the LHC of PS I, is used for carrying out work on the principle of the cyclic phosphorylation, as well (Oquist and Huner, 2003). Besides the changes on the level of both photosystems, the cells of plants, tolerant to low negative temperatures synthesize also cryoprotectors, like sugars, proline etc., that are osmotically strong compounds and can bind free water, thus lowering the freezing point of water and stabilizing the proteins (Dexter, 1933; Anderson, 1944; Sakai,

1962; Levitt, 1980). Apart from that, especially in tree species and plants, tolerant to very low negative temperatures, are synthesized the so-called antifreeze proteins, that obstruct the nucleation of ice crystals, thus enabling plants to survive very low temperatures (Griffith et al., 1997). The process, known as cold acclimation is of extreme importance for most cold tolerant plants for their survival at low temperatures (Weiser, 1970). They acquire resistance by inducing dormancy and dehydration of the cell, because the formation of ice crystals is reduced when the content of free water is low. ABA is the main signal for these plants to enter the phase of acclimation (Mantyla et al., 1995). As a stress hormone, ABA is synthesized and accumulated in higher amounts after treating with low temperatures. Some plant species are able to withstand up to -40^{o}C after acclimation with low (chilling) temperatures or exogenic treatment with ABA (Gusta et al., 1996).

02.02. Response of the photosynthetic apparatus to low temperatures – photodamage

- **ROS as the main damaging factor in the conditions of cold stress**

ROS are highly reactive forms of oxygen and they accumulate after different types of stress (biotic or abiotic), damaging the components of the cell with their action: they oxidize the lipids, damage the DNA and have a negative impact on the physiological process in the cell. ROS form mostly in the chloroplasts, mitochondria and peroxisomes. Mitochondria do not take so big part in ROS formation in plant cells, as compared with animals, probably because of the alternative oxidase enzyme (AOX), which is a concurrent for the substrate of the cytochrome bc_1 complex (Purvis, 1997; Apel and Hirt, 2004). There is a balance between the formation and degradation (neutralization) of ROS in the normally functioning cell. However, when the organism is a subject of stress, this balance is disturbed and ROS start to form inside the organism.

Regardless of the highly thermodynamic reacting ability of the molecular oxygen (O_2), reactions, performed with it require very high activation energy, because of the existence of free π electrons with parallel spin in the main triplet state of the molecule. According to Pauli's principle, electrons with antiparallel spins only can form a couple. For a reaction with oxygen to take part, one of the two free π electrons has to acquire different spin, for which a certain amount of energy in needed. In specific conditions (stress, high light intensity) however, in the reaction centre of PS II and the antennae complexes as well, a triplet chlorophyll forms, that is capable of producing singlet oxygen ($^1O_2$) (Fig. 1). It can be reduced more easily than triplet oxygen and can produce ROS that can bring serious damage

to the plant cell, if they are not neutralized. $^1O_2$ can easily react with proteins, lipids (and mostly with the polyunsaturated fatty acids), pigments (pigment bleaching), and to deactivate PS II by degradation of the protein D_1 (Prasil et al., 1992; Aro et al., 1993). Furthermore, when enough energy is available, oxygen can be reduced directly to a superoxide ion ($O_2^{-\bullet}$) and after that to a peroxide ($O_2^{-2\bullet}$), accepting electron at each of the subsequent steps in the process of ROS formation. After that, these ROS can be protonated until a peroxy radical, hydrogen peroxide and water are formed (Fig. 2). A detailed scheme of the formation and reduction to water of ROS in the chloroplast and peroxisome is given in Fig. 2.

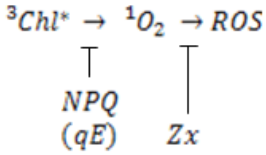


Fig. 1 The main pathway of generation and inhibition of ROS inside PS II. Zx – zeaxanthin (bound to the membrane or in a complex with LHCII) functions synergistically with the tocopherols; qE – non-photochemical quenching (NPQ) with the main participation of PsbS, the first and the fastest phase from the NPQ

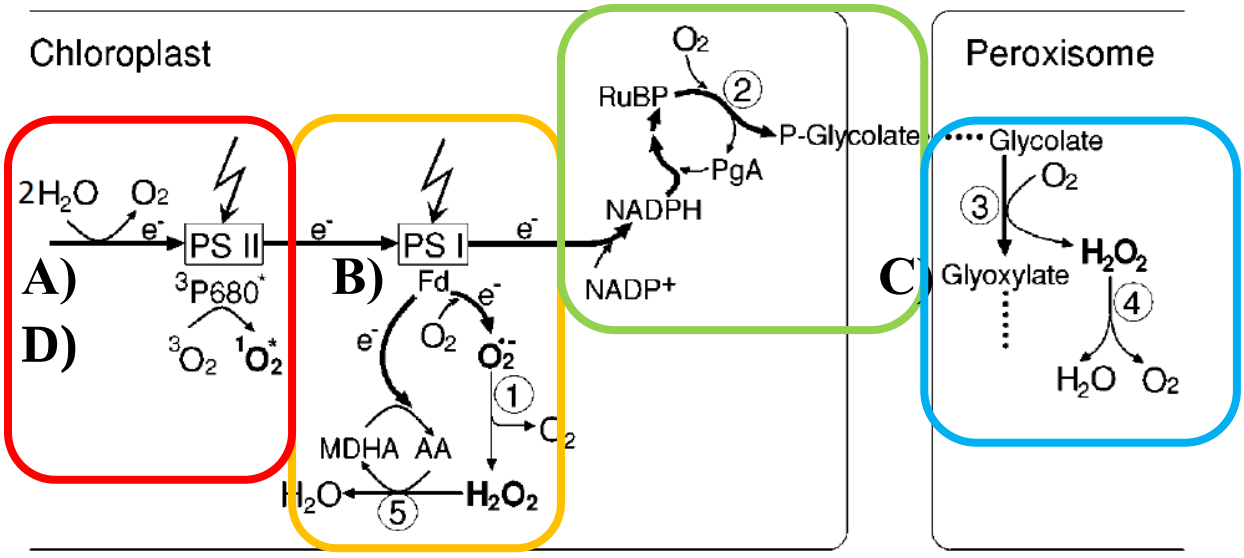


Fig. 2 Processes of ROS generation and their reduction to water in the chloroplast and the peroxisome. A) Singlet oxygen formation ($^1O_2^*$) in PS II. $^3P680*$ – triplet state of the chlorophyll molecules in the reaction centre of PS II. $^3O_2$ – triplet state of oxygen;

B) Photoreduction of oxygen to superoxide radical ($O_2^{\cdot-}$) by the FeS cluster of PS I. The superoxide radical is reduced to hydrogen peroxide with the help of the enzyme superoxide dismutase (1). After that the hydrogen peroxide is reduced to water by the enzyme ascorbate peroxydase. In the process a molecule of ascorbate (AA) oxidises do monodehydroascorbate (MDHA); C) Photorespiration – oxygenase reaction of Rubisco (2). PgA - phosphoglycerate; D) Deactivation of the hydrogen peroxide to water and oxygen by the enzyme catalase (4) in peroxysomes. (Apel and Hirt, 2004).

After the so-called photooxidative damage in plants very large amounts of ROS are produced. When plants are in conditions of light intensity, higher than their capability for assimilation of CO_2, an overreduction (absorbance of electrons) of their electron-transporting components occurs. The latter leads to inactivation of PS II and inhibition of the photosynthesis because of the accumulation of the forementioned ROS. A detailed view shows, that in the process of photooxidative damage the unused additional energy leads to the formation of singlet oxygen from PS II and superoxide ions (radicals) of oxygen from PS I (Fig. 2 A; B) (Asada, 1999; Krieger-Liszkay, 2004). Plants that have adaptation abilities for growth and development in abiotic stress conditions are often a subject to the consequences of the photooxidative damage. These plants tolerate better this type of damage, mainly because of their ability for quenching and neutralization of ROS (Oquist and Huner, 2003; Apel and Hirt, 2004).

The first way of dealing with ROS is diminishing the possibility of their production. This takes place when processes are being activated, that consume the excess oxygen even before it turns into ROS. Three processes assimilate the oxygen inside chloroplasts: I) the oxygenase reaction of Rubisco (or the so-called photorespiration, which is the basic process, assimilating the excess oxygen inside chloroplasts) (Fig. 2 C); II) the direct reduction by PS I (photoreduction – that occupies up to 30% of the electron transport in conditions of intense light) (Fig. 2 B). The next mechanism for suppression the action of ROS at the place of their formation is the quenching of the triplet chlorophyll forms in the content of PS II. The triplet chlorophyll forms, which are a structural part from the antennae complexes are denoted as ^{3}Chl and $^3P_{680}$* is the triplet chlorophyll from the reaction centre of PS II. $^3P_{680}$* can form in two ways, firstly by change in the spin of the electron from singlet oxygen and secondly by reactions of charge recombination in PS II (Krieger-Liszkay, 2004). The first mean for formation of triplet chlorophyll (^{3}Chl) is unique for the antennae complexes, whereas the second process is mainly for the formation of $^3P_{680}$* in the reaction centre of PS II. Charge

recombination normally occurs at the stage of the photochemical reactions from the light phase of photosynthesis. The trapped quantum of light energy from the reaction centre of PS II (P_{680}) is used up in the process of separation and accumulation of electric charge in the components of the electron-transporting chain, and after that is utilised to create chemical potential from both sides of the thylakoid membrane.

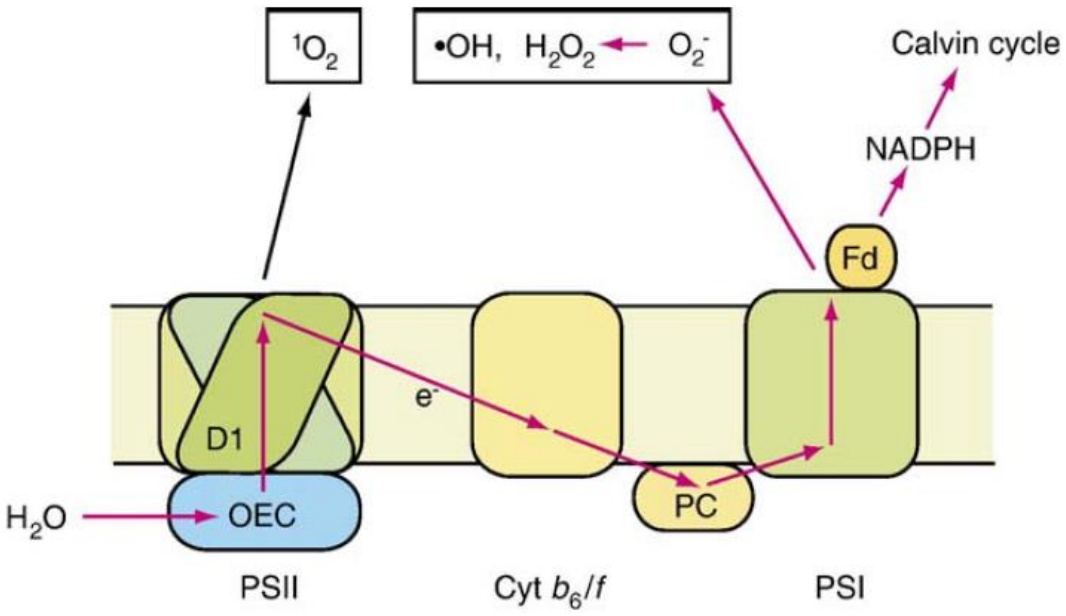


Fig. 3 Diagram for ROS formation in both photosystems. The transfer of energy from chlorophyll in triplet state to the molecule of oxygen leads to formation of a singlet oxygen molecule ($^1O_2$). The reduction of oxygen from the acceptor side of PS I, as a result from photosynthetic electron transport leads to production of superoxide ion (O_2^-), which transforms afterwards to hydrogen peroxide (H_2O_2) and OH. Formation of ROS is induced by strong light and a decrease in the fixation of CO_2. Red arrows represent transfer of electrons, and the black ones are transfer of energy. Fd, Ferredoxin; OEC, the oxygen-evolving complex; PC, plastocyanin. (Nishiyama et al., 2006)

This occurs because of the property of the chlorophyll molecule from the reaction centre to absorb a quantum of light, switching itself into a higher energetic state of a singlet chlorophyll form (P_{680}*). During this process one of the coupled electrons from the porphyrin ring "jumps" to an orbital with higher energy. This state of the chlorophyll molecule lasts for about several nanoseconds, which is enough for reaction of charge separation between the components of PS II to be carried out; more specifically, the chlorophyll from the reaction centre, pheophytin and finally, the primary quinone acceptor (P_{680}*Pheo –> P_{680}^+Pheo$^-$ –> $P_{680}^+Q_A^-$). After the charge separation between the reaction centre and Q_A there is a

probability for the reverse recombination to pheophytin to occur. Since the state $P_{680}^{+}Q_A^{-}$ is relatively continuous, a high probability exists that the electron, which came back to $P_{680}^{+}Pheo^{-}$ to have changed its spin and then the formation of triplet state $^3[P_{680}^{+}Pheo^{-}]$ could be possible (Durrant et al., 1990). Several factors facilitate this process, mostly the change of redox potential of Q_A after stress, whereas some herbicides like DCMU and phenol photosynthetic inhibitors affect it positively or negatively (Keren et al., 1995, 1997; Nakajima et al., 1996). The overreduction of the photooxidative components, followed by blockage of the photochemical reactions also increases the possibility for charge recombination and formation of $^3P_{680}$. For example the accumulation of plastoquinone in its reduced form can obstruct the movement of electrons further away, which creates favourable conditions for recombination (return) of the charge to the reaction centre of P_{680} (Krieger-Liszkay, 2004).

Plants also possess mechanisms for neutralising of the already created triplet forms of chlorophyll, as well as singlet oxygen. Carotenoids, associated with the antennae complexes can directly quench ^{3}Chl and $^1O_2$, forming triplet carotenoid, which dissipates the energy in the form of heat. However, this can only occur when carotenoid molecules are in a direct contact with ^{3}Chl and $^1O_2$. If the space between them is higher than the Van der Waals distance (3.6 Å), the electron orbitals cannot overlap and neutralization of active forms of chlorophyll and oxygen cannot occur. Inside the reaction centre of PS II there are two molecules β-carotene, but nevertheless, the distance between them and the reaction centre is kept too large because of the high oxidative ability of P_{680}^{+}. This fact represents an obstacle for the direct deactivation of triplet chlorophyll inside the reaction centre. Perhaps only $^1O_2$ could be neutralised by the carotenoids from the PS II (Telfer, 2002). Therefore, only the direct quenching of $^1O_2$ can be realized there, mainly with the active participation of the tocopherols (Trebst et al., 2002, 2003; Kruk et al., 2005). If $^1O_2$ is not neutralized, it induces the enzymatic degradation of the D_1 core protein, which is one of the two main components of PS II and this leads to inhibition of photosynthesis (Fig. 4). The degradation of D_1 inhibits the function of P_{680}, including the formation of $^1O_2$. This is considered to be an effective system for the deactivation of the $^1O_2$ itself (Trebst, 2003). Lately, the damaged and degraded D_1 protein is synthesized *de novo* and PS II is restored in the cycle of damage and repair of D_1 (Prasil et al., 1992; Aro et al., 1993) (Fig. 4). Reactive oxygen species, mainly superoxide ions can also form in PS I (Fig. 3) (Apel and Hirt, 2004; Nishiyama et al., 2006). This occurs at the ferredoxin acceptor of PS I, which is a very strong reductor and can easily reduce the oxygen molecule. The reduction of oxygen in PS I is a concurrent process to the reduction of

$NADP^+$. In stress conditions, when the fixation of CO_2 is low and not enough amount of NADPH is utilized, the reduction of oxygen from PS I can be a reason for the formation of reactive oxygen species (Szarka et al., 2012). The superoxide radicals, produced in PS I are deactivated mostly from the enzymatic system of plants, including the enzymes superoxide dismutase and ascorbate peroxidase (Asada, 1999).

In conditions of oxidative stress, plants activate their protection mechanisms against accumulation of ROS. These mechanisms include the increase of the antioxidant activity in an enzymatic or non-enzymatic way. The first type of antioxidant protection includes the enzymes superoxide dismutase, which deactivates the superoxide ions, the ascorbate peroxidase, deactivating the hydrogen peroxide by the means of the ascorbate as a cofactor and the enzyme from the peroxisomes catalase that also deactivates the hydrogen peroxide formed inside the peroxisome (Apel and Hirt, 2004). Non-enzymatic antioxidant defence is realized by the increased synthesis of antioxidant compounds: tocopherol, ascorbate, glutathione (Szarka et al., 2012). The other photosynthetic defence mechanisms include the non-photochemical quenching and the xanthophyll cycle (Jahn et al., 2012). The non-photochemical quenching (NPQ) appears to be the main protective mechanism against the oxidative stress as a result from the excessive light energy (Krause and Weis, 1991). NPQ can be divided to several processes or stages in relation to the period of their activation and the duration of their action (Lambrev et al., 2010; Nilkens et al., 2010). qE (from **e**nergy dependent **q**uenching) denotes the quickest stage (Krause et al., 1982) and includes the activation of the dissipation of excessive energy from the protein PsbS (Li et al., 2000). This stage activates during several seconds and it depends on the accumulation of the proton gradient (ΔpH), thus it is also called ΔpH dependent NPQ (Li et al., 2002; Li et al., 2004).

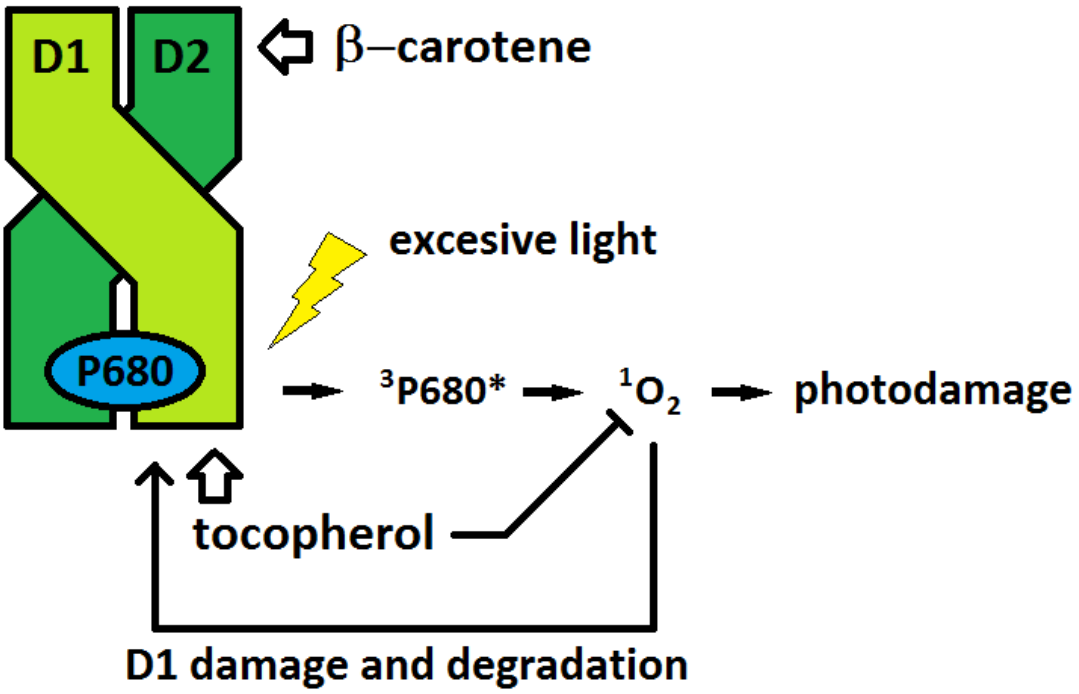


Fig. 4 Diagram of photoinhibition of PS II; formation of reactive oxygen species and the involvement of tocopherol in photoprotection of D1. Carotenoids do not participate directly in the photoprotection of D1, but this is realized by tocopherol. They quench the singlet oxygen, which is a reason for damage and activation of a cycle from the enzymatic degradation and repair of the protein D1. Singlet oxygen is molecule with high reactivity, capable of oxidation of many other components of the photosynthetic apparatus, the unsaturated fatty acids from the membranes being oxidised most strongly. (Wide arrows show the location of the molecules of β-carotene / tocopherol in PS II)

02.03. Plant pigments and antioxidants (terpenoids) as protectors against cold stress

Terpenoids, including the carotenoid pigments, chlorophylls, as well as tocopherols and quinones (plastoquinones) are amongst the main and essential components of every thylakoid membrane. In a wider sense, including the species outside the plant kingdom, biological membranes without terpenoids do not exist in practice (Ourisson et al., 1994).

Thylakoid membranes are exceptionally sensitive and thermolabile, compared to other biological membranes. In many literature data the important significance of tocopherols together with zeaxanthin in stabilising and protecting the membranes from oxidative damages is considered. (Tardy and Havaux, 1997). Some authors attribute to tocopherol the effect, similar to the effect of cholesterol on the biomembranes (DellaPena, 2003).

Carotenoid pigments

The carotenoid pigments, being an important part of the chemical composition of almost every plant, and many other organisms as well, have endogenous content and metabolism, which is exceptionally constant in higher plants (Bungard et al., 1997). The main two functions of carotenoids in plants are related to the light phase of the photosynthesis and the protection against photodamage (Holt et al., 2005). They act as light-harvesting pigments that are the first to capture the light energy and, consequently aim it to the reaction centre of both photosystems by the chlorophylls *a* and *b*. Furthermore, they act as photoprotectors, filtering the short-wave radiation, quenching the triplet chlorophyll forms, neutralising the ROS that were formed (Li et al., 2009; Triantaphylides and Havaux, 2009). Thanks to their specific structure they participate in sustaining the physico-chemical properties of biomembranes in stress conditions and protect them against oxidation (Woodall et al., 1997; Gruszecki, 1999, 2004). The main and most important chemical property of carotenoids is their pigment property for absorption of light with a certain wavelength due to their system of multiple (over 9) conjugated double bonds (Gruszecki and Srzalka, 2005).

The two most important carotenoids, present in most of the plants are the lutein and β-carotene. β-carotene has orange-red colour. It has non-polar molecule, containing two β-rings on both sides of the molecule. Like all carotenoids (and xanthophyll) molecules, β-carotene is also a tetraterpenoid, which means its molecule contains 40 carbon atoms. This is so, because biosynthetic pathway of carotenoids begins from 8 molecules of isoprene, binding to two molecules of geranylgeranyl pyrophosphate (van Arnum, 1998) and after that lycopene forms, starting the metabolism of carotenoids and xanthophylls. Being a non-polar molecule, β-carotene is located inside plant membranes and contributes to their fluidity (Strzalka and Gruszecki, 1994). Therefore, its spatial arrangement inside membranes is not so well-defined, like the xanthophylls for example, but it can form different angles with the surface of the membrane (Van de Ven et al., 1984; Gruszecki, 2004).

The lutein is present in a significant amount in higher plants (Johnson et al., 1993; Bungard et al., 1997), whereas in some of them (including *Arabidopsis*) has the highest content amongst the carotenoid pigments (Havaux et al., 2007). According to the same authors its content is between 30-60% from the total content of the carotenoid pigments in higher plants. Carotenoid pigments are tightly related to photosynthesis and most of them are exceptionally important in the formation and function of the antennae complexes (Standfuss et al., 2005; Dall'Osto et al., 2006). A large percentage of the carotenoids *in situ* (from 80 to

95%, according to some studies) are bound to protein complexes and their dissociation from the proteins occurs very rarely, as in the case of the de-epoxidation of violaxanthin to zeaxanthin during the xanthophyll cycle (Tardy and Havaux, 1997). Lutein is a key structural component in the largest subunits of the light-harvesting complexes and, more specifically Lhcb1-3, which is a part from the trimer complex of LHCII (Liu et al., 2004; Standfuss et al., 2005). Each one of the subunits of LHCII is composed of a polypeptide chain, containing three trans-membrane α-spirals and multiple chlorophyll molecules (8 molecules of chlorophyll *a* and 6 molecules of chlorophyll *b*) are associated to each subunit (Fig. 5). In addition, four xanthophyll molecules are part of the structure of these subunits and each of them binds to a strictly specific place. Two of these four xanthophylls are lutein molecules. In addition, lutein is essential for the correct three-dimensional folding of the antennae proteins (Plumley and Schmidt, 1987; Paulsen et al., 1990). If not sufficient amount of lutein is available, the subunits can bind with other xanthophylls such as zeaxanthin and violaxanthin, but the formed complex is too unstable and its trimerisation is inhibited (Jahn et al., 2001; Dall'Osto et al., 2006; Werner et al., 2006). These facts show the important significance of lutein in the structure of the photosynthetic apparatus. The trimeric subunits of the light-harvesting complex contain one molecule neoxanthin and a molecule from the xanthophyll cycle (zeaxanthin or violaxanthin). The content of neoxanthin is relatively constant in higher plants (about 9-14% of total carotenoid content) (Johnson et al., 1993; Bungard et al., 1997). Other antennae proteins also include xanthophyll molecules in their composition, although in different amounts. For example, large proteins from LHCII and LHCI bind 14 chlorophyll molecules, however chlorophylls are comparatively less in the monomeric proteins from LHCII, Lhcb4-6 (CP24, CP26, CP29). Furthermore, the proteins from LHCII can bind up to 4 xanthophylls, whereas the other antennae proteins can bind two or three (Standfuss et al., 2005; Amunts et al., 2007).

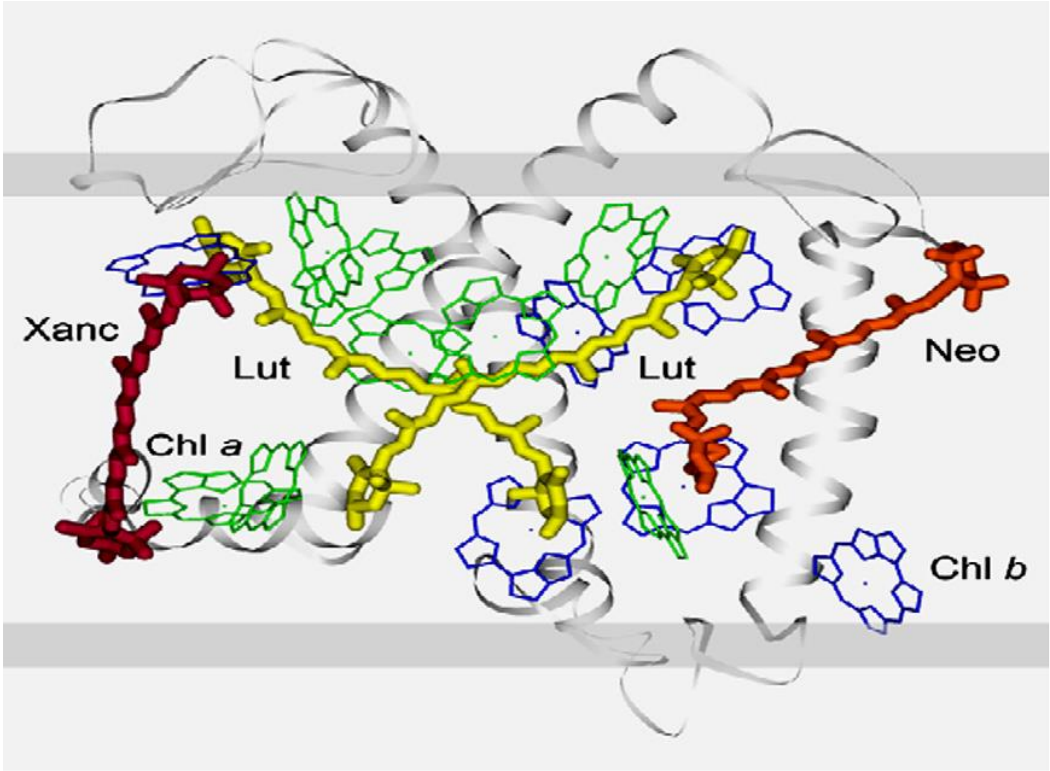


Fig. 5 Crystallographic structure of one of the trimeric subunits of LHCII. Chl. a has a green colour, chl. b is blue. Both lutein molecules are coloured in yellow, neoxanthin is orange, and the violoxanthin/zeaxanthin is dark red. (Jahn et al., 2012 no Liu et al., 2004)

Perhaps one of the most important functions of lutein, concerning the photoprotection is quenching of the triplet chlorophyll forms (Dall'Osto et al., 2006; Mozzo et al., 2008). These authors have confirmed, that the neoxanthin and violaxanthin are much less effective in quenching the triplet forms of chlorophyll and that the most effective is the quenching by lutein, when it is associated with the antennae proteins. The substitution of the lutein inside antennae complexes with other type of xanthophyll leads to highly increased photoinhibition. Other function of lutein is to capture the light energy and transport it to the chlorophyll molecule (Siefermann-Harms, 1985). Nevertheless, the violaxanthin is more effective light-harvesting pigment than lutein and can entirely substitute it in this function (Pogson et al., 1996; Kalituho et al., 2007). The lutein-epoxide, which is a part from the so-called lutein cycle captures light energy also more efficiently than lutein (Matsubara et al., 2007; Garcia-Plazaola et al., 2007; Matsubara et al., 2009). It appears, that some xanthophylls are self-replaceable, but this substitution is limited to specific functions only (lutein-violaxanthin) or to a certain degree (as in the case with lutein and zeaxanthin in structural relation) (Jahn et al., 2012). The function of xanthophylls is determined not only by their chemical composition as well as structure, but also by their binding in complexes with proteins (the antennae proteins).

It is widely accepted, that lutein acts like quencher of the excited singlet forms of the chlorophyll, but this function is not related to the non-photochemical quenching (Pogson et al., 1998; Lokstein et al., 2002). According to some new literature data, the role of lutein in this type of quenching is insignificant, because the excessive unused energy is re-distributed amongst the chlorophyll molecules (Muller et al., 2010).

Zeaxanthin is one of the three pigments from the xanthophyll cycle (along with the violaxanthin and antheraxanthin). This cycle acts in the quenching of ROS and protects from photodamage after abiotic stress and intense light (Sapozhnikov et a, 1957; Yamamoto et al., 1962). The zeaxanthin itself is extremely important for the normal function of thylakoids and reactions from the light phase of photosynthesis after photodamage by certain types of abiotic stresses (Havaux and Niyogi, 1999). Due to its structure, zeaxanthin increases the membrane fluidity, attaching to both sides of the membrane with the two polar ends, whereas the non-polar part remains in the centre of the membrane (Tardy and Havaux, 1997). Thus, it increases membranes stability and strengthens the impermeability of the membranes to oxygen, protecting their sensitive hydrophobic parts from the oxidative action of ROS (Gruszecki and Strzałka, 2005). These properties make its physiological action very similar to the cholesterol inside membranes of animal cells. The latest literature data (Demmig-Adams et al., 2012) recently showed, that carotenoids together with tocopherol also affect the synthesis of oxylipins and oxylipin hormones (jasmonates), deactivating the ROS, which have been accumulated as a result from the negative effect of light. The greatest photoprotective properties of all carotenoid pigments, are attributed to the zeaxanthin (Havaux et al., 2007). This is mainly due to its chemical structure that includes two polar rings with function, described briefly earlier. Zeaxanthin molecule has a non-polar body with a system of 11 conjugated double bonds, explaining its light absorption properties. In contrast, lutein and antheraxanthin have both 10 such double bonds, violaxanthin has nine and neoxanthin has seven. It is considered, that the higher the amount of these double bonds, the greater the photoprotective properties of the molecule (Woodall et al., 1997; Gruszecki, 1999, 2004; Gruszecki and Strzalka, 2005). These properties are amplified after binding in complex with the subunits of LHCII. There, it can quench directly the triplet chlorophyll and singlet oxygen species, formed in P680. It was found out, however, that zeaxanthin can take part in photoprotection enough actively even without binding to the LHCII (Havaux et al., 2007).

According to the commonly accepted opinion, the increased zeaxanthin content after light stress (combination of temperature stress and photodamage), is due to the reversive

conversion of the violaxanthin into zeaxanthin (de-epoxidation of violaxanthin) as a component from the xanthophyll cycle. In normal conditions and in absence of stress, zeaxanthin turns into violaxanthin by the means of the enzyme zeaxanthin epoxidase (ZEP or ABA1, mutant forms of this enzyme in *A. thaliana*). This reaction occurs in the stroma of chloroplasts at pH 7 and in the presence of O_2. In stress conditions, violaxanthin is converted to zeaxanthin by the reverse reaction of de-epoxidation, which takes place in the lumen of the thylakoid membranes, at pH 5.2 by the enzyme violaxanthin de-epoxidase. During this reaction the violaxanthin is dissociated from the anthennae complex, and after the formation of zeaxanthin it is possible to be inserted in the light-harvesting complexes again, in the appropriate conditions (Tardy and Havaux, 1997). It is well-known, that zeaxanthin has a greater capacity than violaxanthin for neutralization of the ROS, which accumulate in high amounts after freezing stress, especially in combination with photodamage. The presence of free carotenoids inside the membrane leads to increase in its stability and limits the lipid peroxidation (Lim et al., 1992; Sarry et al., 1994; Havaux et al., 1996). Furthermore, the singlet electron forms of zeaxanthin has lower energy, even than chlorophyll, which allows the unused light energy to be quenched non-photochemically (dissipating it in a form of heat) (Frank et al., 1994). This is not true for violaxanthin, whose energetic forms are higher than chlorophyll and thus it can act as a light-harvesting pigment, but not as a quencher of energy. In some plants, a conversion cycle of carotenoids exist, similar to the xanthophyll cycle, when the light is in excess. It is called the lutein cycle and it includes the participation of lutein, which consequently epoxygenates at low intensity of light, and de-epoxygenates at high intensity of light, similarly to the conversion of zeaxanthin to violaxanthin and vice versa. This demonstrates the role of lutein as a pigment, quenching the unused energy like the zeaxanthin (Bungard et al., 1998).

Tocopherols

Tocopherols are terpenoid compounds that have the function of antioxidants. They are widely distributed in nature and take place in the antioxidant defence system of organisms. α-tocopherol is one of the most well-known of them under the name vitamin E. Tocopherols participate in the neutralization of the reactive oxygen species, mostly the singlet oxygen formed in PS II of thylakoids. Tocopherols are a component from the thylakoids of chloroplasts as well, because they have a lipid nature and their hydrophobic (prenyl) groups can attach to the membranes. They are synthesised from the shikimate pathway in plants and exist in four forms, depending on the number and location of the methyl groups, attached to

their hydroquinone ring (α, β, γ, δ). They induce the formation of jasmonates from the peroxidation of lipids in the thylakoid membranes in plants after stress.

The main function of tocopherols is their antioxidant action. It is realized mostly in quenching and deactivation of the singlet oxygen forms, the lipid peroxyl radicals, neutralizing the heavy metal ions etc. (Szarka et al., 2012). α-tocopherol molecule has the ability to quench (only in physical way by resonance energy transfer) averagely about 120 molecules of singlet oxygen before undergoing oxidation by chemical deactivation (Fahrenholz et al., 1974; Munne-Bosch and Alegre, 2002). The reactive oxygen species, that form after stress attack mainly the membrane system of plant cell, mostly the sensitive thylakoid membranes in chloroplasts. Initially, lipid peroxyl radicals form in this process. α-tocopherol neutralizes these radicals converting itself into tocopheroxyl radical, and lipid peroxydes into lipid hydroperoxides (Kamal-Eldin and Appelqvist, 1996). After that tocopheroxyl radical can be re-generated from the antioxidant system of the cell, a conjugation of tocopherol, ascorbate and glutathione molecules in action of their mutual re-generation (Szarka et al., 2012). The ascorbate and glutathione themselves are very strong antioxidants and have the ability to reduce the hydrogen peroxide, that forms from ROS after stress. In the process their oxidized forms are produced re-generating (reducing) themselves by the means of so-called antioxidant system, the tocopherol being reduced by ascorbate, ascorbate by the glutathione and glutathione by NADPH. However, in case some of these compounds is insufficient amount, tocopherol cannot re-generate and oxidized tocopherol products form that cannot be re-generated to tocopherol (Kamal-Eldin and Apelqvist, 1996). During chemical deactivation of the singlet oxygen tocopherol radicals form as well (tocopherol quinones etc.), that cannot be subjected to re-generation from the antioxidant system of plant cell (Munne-Bosch and Alegre, 2002). Normally, in tolerant to stress plants the content of tocopherol increases significantly, whereas in sensitive plants it decreases after stress (Munne-Bosch, 2005). Furthermore, in tolerant plants the amount of tocopherols decreases after intensive and prolonged stress exposure (Boo and Jung, 1999). Many transgene plants which express high levels of the genes for biosynthesis of tocopherols have increased tolerance to stress, for example increased tolerance to drought in tobacco (Liu et al., 2008), to cold stress in Arabidopsis (Maeda et al., 2006), tolerance to osmotic stress in tobacco (Abbasi et al., 2007). In the last case it was found, that plants with increased expression of α- or γ-tocopherol have different resistance to different types of stress. In the same way in plants, showing a deficit in α-tocopherol a lower tolerance to stress was

observed, for example to heavy metals in Arabidopsis (Collin et al., 2008). The very content of tocopherol in the cell is a result of balance between synthesis, re-generation and degradation. Therefore, regardless of the high antioxidant and protective function of tocopherols, their defensive function is limited by the presence of other antioxidants in the cell and by other defence mechanisms, as well as by the duration and intensity of the stress factor (Szarka et al., 2012).

02.04. Principle of the chlorophyll fluorescence

Photosynthesis is a main physiological function of the most plant organisms (higher plants and algae), cyanobacteria and other prokaryotes. Photoautotrophic organisms separate in this group by their ability to photosynthesise. The separate groups of photosynthesising organisms have differences in their mechanisms of photosynthesis, but nevertheless, its main action is related to the act of capturing the light energy and its conversion to chemical energy of glucose and other chemical compounds. This occurs in two phases, a light and a dark phase. During the light phase a sequence of oxidative-reduction reactions are carried out by the photochemical pathway including one or two photosystems. They create a transmembrane proton gradient, utilized for the synthesis of ATP. During the dark phase organic compounds are synthesized from the fixation of atmospheric CO_2, as well as ATP and NADPH produced during the light phase, by the means of a sequence of metabolic pathways, the main of which is the so-called Calvin-Benson cycle (Taiz and Zeiger, 2006).

During the light reactions in higher plants two photosystems take place, PS II and PSI. Currently large number of methods for analysis of the functional condition of reactions from the light phase of photosynthesis exist, based on the method of the fluorescence of chlorophyll. In this current work, the methods of prompt chlorophyll fluorescence (PF) at 685 nm, giving information for the structural and functional condition of PS II *in vivo*, as well as the modulated 820 nm light reflection (MR820), characterizing PS I *in vivo* were used. Data was obtained with the M-PEA (multifunctional plant efficiency analyser, Hansatech) that can perform measurements by both methods, simultaneously.

Prompt chlorophyll fluorescence (PF)

The method of the prompt chlorophyll fluorescence is based on the specificity of the PS II to emit in the far-red range of the light spectrum (>700 nm) after illumination by light with wavelength of 650 nm (Goltsev et al., 2010, Goltsev et al., 2014). If the illuminated light is registered by a device with a high time-resolution (in the range of micro- and milliseconds) a characteristic curve of the intensity of the emitted fluorescence, described first by Kautsky

and Hirsch (1931) as the transient curve of Kautsky can be observed. In the first several milliseconds, up to 1 sec. the intensity of the fluorescence quickly increases, and then starts to decrease more and more slowly almost to the initial levels of illumination. Due to the specifics of the speed of increase, curves usually are represented in a logarithmic scale. The curves, obtained from the measurement of most plants, growing in normal physiological condition in absence of stress are characterized with three clearly distinguishable phases (or steps) of increase, followed by steps of momentary maximum (relatively constant levels of fluorescence), denoted with the letters J, I and P. They could be seen as a kind of "bottlenecks" of the electron-transport chain, where the charge accumulates for a certain period of time, until it is released to the next components (Gao et al., 2013). The slower phase after them, characterizing with a decrease in the intensity of the fluorescence can also be divided into stages, denoted as P, S and M, but, their analysis is limited in practice (Stirbert et al., 2013). The initial phases give the name JIP-test of the method of prompt chlorophyll fluorescence, which is widely used and can often be found in literature (Strasser and Govindjee, 1992; Strasser et al., 2004).

Induction OJIP-curves (Fig. 6, I) give detailed information about the redox states of PS II. According to the theory of Duysens and Sweers (1963), two states of the reaction centre of PS II exist: open, when the primary quinone acceptor of the reaction centre (Q_A) is oxidized and closed, when this acceptor is reduced (oxidized means deficit of electrons or positively charged, and in the case of quinone this is its neutral form Q_A; reduced means having excess of electrons or negatively charged, Q_A^-). The type of the moment state of the reaction centre of PS II has a direct impact upon the intensity of the fluorescence. When the reaction centre is "open", the light energy turns it into excited state and donates an electron, reducing the primal quinone acceptor of electrons in PS II (Q_A). Thus, light energy will be spent for activation of the photochemical reactions and the fluorescence of the current reaction centre in open state will be minimal. When the reaction centre is "closed", (according to Duysens and Sweers), Q_A^- is in a reduced state and cannot accept an electron from the reaction centre, therefore the energy will be emitted in the form of fluorescence, which will be maximal for this current reaction centre (in "closed" state). Other models for description the states of the reaction centre exist (with the phaeophytin, as the primal acceptor), but the model of Duysens and Sweers is commonly accepted and results from the current work will be analysed according to it.

The phases (stages) of the transient JIP-curve are defined on the basis of the model of Duysens and Sweers for "opening" and "closing" of the reaction centres. Most devices for measurement of prompt chlorophyll fluorescence calculate these stages at fixed intervals after the start of the illumination with 685 nm. Modern devices have very high time-resolution, and the first value of the fluorescence intensity could be determined after 0.01 ms. This value is denoted with the letter O on the curve, and the intensity of fluorescence for this time, as F_O. Usually, for the purposes of most studies before the illumination with actinic light, plant samples stay for a certain period of time in the dark, called "dark adaptation". During this period the reaction centres of PS II relax to open state. This is the reason for the initial phase of fluorescence to be minimal. The next important value of the transient curve is the last point from the transient JIP-curve (P), located at about 300 ms from the start. Normally, its intensity of fluorescence F_P is identical to the maximal intensity of fluorescence F_M (in plants at optimal physiological condition). Most of the reaction centres in PS II are closed then and this is the reason for the fluorescence in this point to have a maximal intensity. In the period from O to P cause for the lower intensity of fluorescence is the photochemical quenching. Electrons, donated from the reaction centres of PS II gradually fill up the photochemical pathways from the light reactions of photosynthesis. This leads to closing of the reaction centres and a larger part of the energy is redirected as fluorescence. After the point of maximal fluorescence F_M, a period of a gradual decrease in the fluorescence intensity follows. The forces, that act during this period include activation of the later stages of photosynthesis such as the cycle of Calvin-Benson, as well as other processes, differing from the photochemical reactions in photooxidative chains and were denoted already by Kautsky with the common name non-photochemical quenching. They include the thermal dissipation that increases gradually, as well as the re-grouping of the light-harvesting complexes and thylakoid membranes after filling up the plastoquinone pool. Clearly distinguishable stages of relative constant intensity of fluorescence, denoted with J (at 2 ms) and I (at 30 ms) exist between the points of minimal (initial) O and maximal fluorescence (P). They reflect the reduction of the primary electron acceptor of PS II Q_A -> Q_A^- as well as the plastoquinone pool PQ -> PQH_2, respectively. Between the O-J phase of prompt fluorescence kinetics, other two harder to distinguish stages L (150 μs) and K (300 μs) exist (Strasser et al., 2004; Gao et al., 2013). They reflect structural and functional specifics in PS II. The point L relates to the possibility for energy transfer between the components of PS II. K depends on the level for electron transfer at the donor and acceptor places of PS II.

The differences, obtained from subtraction of the intensities F_O, F_J, F_I at different stages of the transient curve to the intensity of the maximal fluorescence F_P (F_M) are denoted as a variable fluorescence in the corresponding point of the time scale. They describe the so-called energy fluxes (Pallotin, 1976). When relating these fluxes to each other the so-called parameters of prompt chlorophyll fluorescence can be obtained. For example, the difference between the minimal and maximal fluorescence ($F_M - F_O = F_V$) reflects the ability of the reaction centres from PS II to capture light energy and is often denoted as TR_O (trapped energy flux). In a similar way the difference $F_M - F_J$ reflects the electron transport beyond Q_A. It could be found also as ET_O (electron transport flux). $F_M - F_I$ relates to the reduction of PS I by the electrons, coming from PS II, as well as the reduction of the next components (Fd, $NADP^+$). Therefore it is denoted as RE_O (reduction of end acceptors of PS I acceptor side). When the relations of the forementioned differences to F_M are calculated, the so-called quantum yields are obtained. The maximal quantum yield of the primal photochemical reaction is the first of these parameters. It shows the amount of energy, trapped by the reaction centres of PS II in the start of the light reactions and could be found from the formula: $\varphi_{Po} = TR_O /ABS = F_V/F_M = (F_M-F_O)/F_M$. The next of the quantum yields reflects the amount of transported electrons from PS II to PS I. It is called the quantum yield of the electron transport: $\varphi_{Eo} = ET_O /ABS = (F_M-F_J)/F_M$. The last of the parameters, describing quantum yields is the so-called quantum yield of the reduction of end acceptors of PS I and, as its name proposes it shows the degree of reduction of end electron acceptors of PS I. It is expressed with the equation $\varphi_{Ro} = RE_O /ABS = (F_M-F_I)/F_M$. These parameters are amongst the most widely used in fluorescent analysis.

Other parameters, often measured in studies with prompt chlorophyll fluorescence are the quantum probability ψ_o for movement of electrons beyond Q_A and the performance indexes, as well as the parameters, reflecting the energy dissipation. The performance index on absorption basis (PI_{abs}) combines the forementioned quantum yield parameters (Goltsev et al., 2012) and reflects the condition of components from PS II and intersystem components (Strasser et al., 2004, 2010). Besides them, the total performance index (PI_{total}) includes the relative amount of chlorophyll in a reaction centre and is a parameter, describing the overall condition of every photooxidative component (Strasser et al., 2004, 2010). Energy dissipation could also be described by the parameters DIo/RC and φ_{Do}. They reflect the dissipation flux and the quantum yield of energy dissipation, respectively. Their application includes the

measurement of dissipated or unused energy from the plant during light phase of photosynthesis and could be useful for describing the damages, caused by stress.

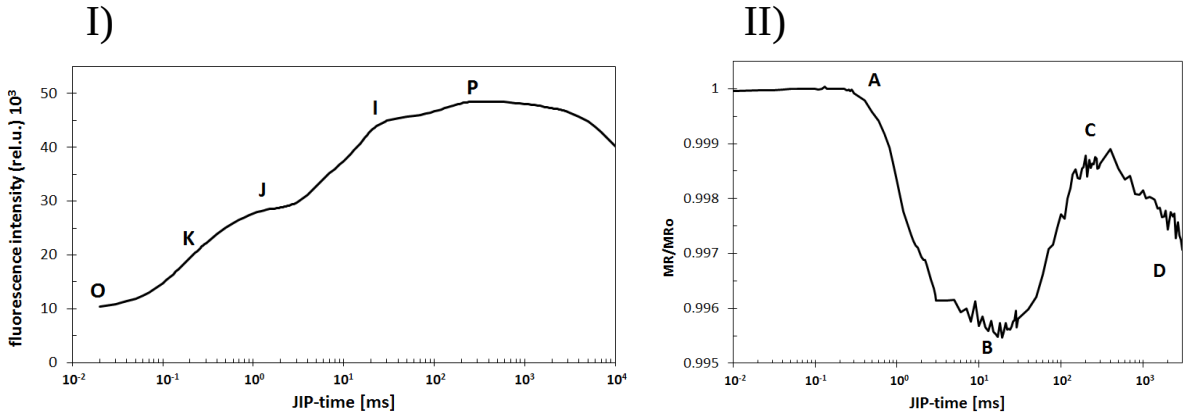


Fig. 6 I) A typical JIP-curve of the prompt fluorescence. The curve was displayed on a logarithmic scale from 0.01 μs to 10 s. The characteristic points O, K, J, I and P, could be seen, which are taken in the calculations of parameters of the fluorescence. The curve represents the prompt chlorophyll fluorescence of A. alpina from Rila mountain, at temperature of 22°C.

II) A typical curve of the modulated 820 nm light reflection. The stages of oxidation (A-B) and re-reduction (B-C) could be seen, as well as the last phase of oxidation C-D, matching with the stages after the point P from the JIP-curve. The curve represents the prompt chlorophyll fluorescence of A. alpina from Rila mountain, at temperature of 22°C.

Modulated 820 nm light reflection (MR_{820})

The next method, available with the M-PEA performs measurement of the redox states of the reaction centre of PS I and plastocyanin. It is called the modulated 820 nm light reflection measurement, because an 820 nm light pulse is emitted and the reflected light in this process is measured. In their oxidized state, $P700^+$ and PC^+ have a greater ability to absorb light in the 820 nm range, thus a lower amount of the emitted light by the device is reflected back, as compared with the reduced state of these two components of the photooxidative chain. The transient curve (Fig. 6, II), obtained with this method has a characteristic downwards course in the period about 1 ms after the start of illumination, causing the oxidation of P700 and PC. After about 15-20 ms the curve changes its course to upward direction, which corresponds to the second reduction (re-reduction) of $P700^+$ and PC^+. During the period around 300 ms after the start of illumination, corresponding to the maximal

fluorescence of the reaction centre of PS II, the signal returns to its values before the oxidation and then starts decreasing again. It is worth noting, that M-PEA can measure simultaneously both types of fluorescence (PF and MR). When both curves are compared (Goltsev et al., 2010), it can be seen that the downward part of the MR curve (A-B phase from Fig. 6 II the oxidation of PC and the reaction centre of PS I) matches with the J-phase of PF, whereas the rising part of the curve (the phase B-C from Fig. 6 II of the reduction of PS I) matches with the stage I-P in PF. The MR-curve in its upper part corresponds to the inclusion of the newly synthesised compounds (ATP, NADPH) from the light phase in the Calvin-Benson cycle (the phase of the carbon reactions) (Strasser et al., 2010). By reading the data, obtained from the measurement of the activity of both photosystems, the processes of oxidation and reduction taking place during the light reactions, could be followed. During the period of the first two milliseconds from the start of illumination with actinic light, a donation of electrons from both photosystems begins. The time, however, is not enough for the electrons to leave PS II and, as a consequence, reduced forms of Q_A accumulate, which could be seen as an increase of the fluorescence until the J peak. Meanwhile, PS I oxidises as a result from absorption of actinic light, but at the same time it is reduced from the electrons, coming from the plastocyanin or even the plastoquinone pool. In fact, the degree of oxidation of the plastoquinone pool, changes under stress, as well as different conditions of the environment, and is related to the reduction of PS I and the oxidation of PS II. Depending on the speeds of the reactions of reduction and oxidation, the faster process predominates. The reaction of plastocyanin oxidation and reduction of P700 has a duration of about 200 μs, which is exactly the same, as the initial stationary phase of the MR-curve. After that the electrons deplete and P700 oxidises quickly about 2 ms after the start of the illumination. This speed of oxidation depends on the functional state of PS I. The rate of the reactions after ferredoxin is speed-limiting in this case. After the reduction of Q_A by PS II, which is reflected on the J peak in the PF-transient curve, the slower reduction of PQ occurs. For a molecule of PQ to be reduces, two electrons are necessary, and a pool of about 7-10 molecules functions in the intersystem electron transport chain, so the process of pool reduction is slower, as well. The dynamic of this process is shown as increase of the fluorescence up to the point I. In the same time P700 remains in oxidized form. After the PQ-pool is reduced and enough electrons are accumulated, they pass to P700, reducing it. This reduction can be observed as increase in the MR-curve. The process is much slower than the oxidation, because it reflects the occurrence of two concurrent processes that are the oxidation of P700 when it absorbs light quanta from PS II and the reduction of $P700^+$ by the reduced PQ. After electrons from PS II

reach PS I, the reduction of end electron acceptors such as the NADPH reductase starts and the cycle of Calvin activates. This releases space for other electrons to enter, which is shown as a decrease in the PF and MR curves movement. A large part in this decrease in the fluorescent curves takes the re-organisation of PS II from state 1 (granal) to state 2 (lamellar) (Stirbert et al., 2013). It activates few seconds after illumination with saturating actinic light and is related to the phases of the transient PF curve after P.

02.05. Plant hormones as regulators of the tolerance to cold stress

Abscisic acid (ABA)

ABA is responsible for the induction of dormancy in plants or decreased living activity after the effect of stress. It is the main hormone, together with ethylene, inducing dormancy state in the perennial plants from the moderate climate during the winter season, as well as in seeds during maturation (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005). One of the main and most important function of ABA is the closing of the stomata in conditions of stress (Beardsell and Cohen 1975). Thus, ABA is considered a hormone responsible for the tolerance of plants to abiotic stresses, mostly to drought (Swamy and Smith, 1999; Mahajan and Tuteja, 2005), as well as to cold stress (Gusta et al., 1996). Besides seed maturation and induction of dormancy in plants in stress conditions, reports for the action of ABA during other processes of growth and development in plants exist, mostly during the reproductive phase, such as accumulation in the reproductive organs in significant amount, but also during the phase of growth in much lower content.

ABA metabolism starts in the chloroplasts from the isopentenylphosphate, partially overlaying the biosynthesis of carotenoid pigments and xanthophylls. An important part of the biosynthetic pathway of ABA is the epoxidation of zeaxanthin to trans-violaxanthin, which itself is a step from the xanthophyll cycle and is realized by the enzyme zeaxanthin epoxidase (ZEP/ABA1). After that follows the isomerization of the trans-violaxanthin to 9-cis-violaxanthin and the synthesis of the 9-cis-neoxanthin by the enzyme ABA4. The enzyme 9-cis-epoxycarotenoid dioxygenase catalyses the conversion of xanthoxin from 9-cis-neoxanthin or 9-cis-violaxanthin. Xanthoxin is the first precursor of ABA, having ABA activity. After its synthesis it is transported outside of the chloroplasts. Enzyme xanthoxin dehydrogenase (ABA2) converts the xanthoxin to ABA-aldehyde, which then oxidises to ABA by the enzyme ABA-aldehyde oxidase (AAO3).

ABA can be deactivated temporarily by converting into glucose ester, which is a way to store (transport) ABA to different parts of the plant organism. ABA degrades irreversibly,

first oxidizing to phaseic acid (having partial ABA-activity), and then to dihydrophaseic acid (does not have ABA-activity). An intermediate step from ABA oxidation to phaseic acid is the 8′-OH-ABA, which is unstable and spontaneously converts to phaseic acid (Nambara and Marion-Poll, 2005; Okamoto et al., 2011).

It is well-known, that ABA regulates closing of stomata after drought (Davies et al., 2002). Being a weak acid, ABA dissociates to ABA^- and H^+, in the condition of high pH. ABA can penetrate freely inside cell membranes in its protonated state by the means of the process passive diffusion along the concentration gradient, but when dissociated, it cannot cross the membranes. In normal conditions, in absence of stress, ABA is permanently synthesized in very small amounts in the mesophyll cells. When drought stress occurs, the pH of the mesophyll increases. This leads to dissociation of ABA and is an obstacle for the accumulation of ABA inside cells, because membranes are impermeable to ABA^-. Therefore, large amounts of ABA accumulate in the mesophyll and much larger part of ABA reaches the cells of the stomata and exerts its signal function upon them. It is not completely clear what exactly is the mechanism of stomata closing. Two assumptions for this signal pathway exist, and both include calcium ions as an intermediate messenger in the signal transfer. In generally, when ABA reaches the stomata cells, the intercellular content of calcium ions increases, because calcium leaves the vacuole. After that, calcium activates the potassium ion channels that start pumping potassium out of the cell. This leads to a change in the osmotic potential of the cell, which shrinks as a result of the leaving of the water out of it in the direction of the osmotic gradient. Finally, this shrinking of the stomata cells closes the stomatal aperture (Beardsell and Cohen 1975).

In absence of stress and presence of high photosynthetic activity, ABA accumulates in the stroma of chloroplasts. During light reactions of photosynthesis, protons are being pumped in the thylakoid lumen and therefore pH of the stroma rises. It was mentioned, that ABA is a weak acid and high pH causes it to dissociate and afterwards it loses the ability to cross the membranes. When photosynthetic activity is high, ABA enters the stroma in the direction of concentration gradient, until the content of ABA inside chloroplast and cytosol is equal. The bigger the photosynthetic activity, the more ABA accumulates in chloroplasts. However, when photosynthesis is inhibited by some reason, pH of the stroma decreases, ABA leaves the chloroplasts and its content in the apoplast increases.

- **Cytokinins (CK)**

Cytokinins regulate and participate in the processes of growth and development in plants. They are a large group of phytohormones with adenine nature and include five main groups: the bioactive cytokinins (*trans*-zeatins, isopentenyladenines, dihydrozeatins), the *cis*-zeatins, considered inactive, the precursors of the biologically active forms CK-monophosphates, the transport forms CK O-glucosides and the biologically inactive N-glucosides (Kamada-Nobusada and Sakakibara, 2009). The cytokinins (CK) have diverse functions in plants, the main of them being the stimulation of cell division and growth of the plant shoots (meristem tissues). The ratio between auxins and cytokinins defines the growth of shoots (upper part) or roots (underground part) in the plant. CKs affect the apical dominance (Sachs and Thimann, 1967; Tanaka et al., 2006). Cytokinins affect mostly the mitotic cycle during the activation of cell division, and this is where their name comes from (cytokinesis, gr. action, division of cells). Their activity on processes of division and growth of plant cells stimulates life functions in the plant organism. Cytokinins participate in the formation and development of chloroplasts as well, activating the synthesis of chloroplast proteins and photosynthetic pigments (Huff and Ross, 1975). They take part in the stimulation of the synthesis of proteins in the cell by the means of formation of the polyribosome apparatus (Ananiev et al., 1980; Ananiev et al., 1987). CKs are hormones, which stimulate life activity in plants, in contrast to ABA, a hormone, functioning as an inhibitor on the physiological activity in plants. Both hormones exert antagonistic actions against each other and cause mutually neutralizing effects in many processes (Ananiev et al., 2001; 2002). Their antagonistic effect against ABA is displayed in the overall activation of the living processes in the cell, as well as in the induction of the stomatal opening. As hormones of growth, CKs regulate the photosynthetic activity, affect the circadian rhythms and slow down the processes of aging in plants, as well (Gan and Amasino, 1995). In addition, CKs affect the transport and utilization of metabolites, targeting them from the places of synthesis to the places, where they are needed the most (Mok, 1994; Forde, 2002).

According to some studies, cytokinins act cooperatively with light, in the sense that they exert effect, similar to light and after combining both factors they amplify each other. Newer literature data, however, showed that cytokinins in combination with light tend to damage the plant after a certain stage (accelerated aging process) (Vlckova et al., 2006).

CK metabolism

Cytokinins are synthesised from the mevalonate biosynthetic pathway (MEP) or from the MVA. *Cis*-cytokinins are synthesised from tRNA by degradation of nucleotides. Normally, *trans*-cytokinins are considered to have the highest biological activity. The largest group of cytokinins (tZ, DHZ, iP) are synthesised from the dimethylallyl diphosphate and ATP or ADP to form iP-riboside di- or triphosphates (iPRT, iPRD). This occurs by the means of the enzyme adenosine-phosphate isopentenyl transferase (IPT) that has multiple forms. Their oxidation to *trans*-zeatin ribosides is catalysed by the cytochrome-P450 mono-oxygenase enzymes CYP735A1 and CYP735A2 in *Arabidopsis* (Takei et al., 2004). DHZ and its derivatives are synthesised from tZRMP until the formation of DHZRMP. After that, in order to be converted into their corresponding biologically active forms, their phosphate residues have to be removed as well as their riboside residue. *Cis*-CKs are synthesised by prenylation of their adenine group at the 3' end of the anticodon of tRNA by the enzyme tRNA-IPT (Skoog and Armstrong, 1970; Letham and Palni, 1983; Murai, 1994; Kamada-Nobusada and Sakakibara, 2009). A *Cis/trans* isomerase has been isolated from the endosperm of *Phaseolus vulgaris* (Basil et al., 1993). It is assumed that this enzyme isomerises *cis*- into *trans*-zeatins and vice versa in *Phaseolus*. In other plant species, including *Arabidopsis* and rice, such enzyme has not been found, and the probability for a spontaneous process of isomerization between *cis*- and *trans*-zeatin has been rejected (Gajdosova et al., 2011; Kudo et al., 2012). By using a mutant *Arabidopsis* of both genes for tRNA-IPT, Miyawaki et al., 2006 proved that in *Arabidopsis*, cZ is synthesized by the enzyme tRNA-IPT. Furthermore, the prenyl group of tZ and iP comes mainly from the MEP, whereas this group in cZ is transported from MVA in *Arabidopsis* (Kasahara et al., 2004). The same authors have found, that the product of the gene AtIPT2 in *Arabidopsis*, tRNA-IPT is localized in the cytosol.

The *cis*-CKs were considered biologically inactive for a long time, but now it is known that they have the biological activity for induction most of the processes in the cell, even if lower as compared with *trans*-cytokinins in *Arabidopsis* (Gajdosova et al., 2011). It is not completely clear what is the function of the *cis*-CKs. There are many plants, existing inside different plant groups, that contain mostly *cis*-CKs, as well as plant species, where the *trans*-CKs predominate. During the germination of *Arabidopsis*, for example, *cis*-CKs are in highest amount and immediately after that *trans*-CKs quickly begin to accumulate until the *cis*-CKs reach 5% from the total content. In the offset of the aging process the *cis*-CKs again

start to accumulate (Gajdosova et al., 2011). Three cytokinin histidine-kinases (ZmHK1-3) having different susceptibility to tZ, as well as to cZ were found in maize, in contrast to AHK4 in *Arabidopsis* for example, which is active only to tZ, but not to cZ (Yonekura-Sakakibara et al., 2004; Lomin et al., 2011). The histidine-kinase receptors in maize have also a very high affinity for iP. The biological role of the individual cytokinins, however, still remains unclear. In plant species, that have higher content of *cis*-CKs, their biological activity is also higher. For example in *Arabidopsis*, where *trans*-CK prevail, their biological activity is higher than the activity of *cis*-CKs, in contrast to rice and maize, where the *trans*- and *cis*-CKs have almost the same biological activity (Yonekura-Sakakibara et al., 2004; Lomin et al., 2011; Kudo et al., 2012). In transgenic rice plants with an overexpressed gene for *cis*-zeatin-O-glucosil transferase (cZOGTase) a short phenotype and slowed aging of leaves was observed (Kudo et al., 2012).

CKs and stress

Being a stress hormone, ABA has been paid higher attention, compared with the CKs, concerning the effects of the abiotic stress factors, such as drought and cold stress. As activators of the physiological activity in plants, CK regulate the response to stress of the cell. CKs are the main antagonists of ABA and thus their content largely decreases after stress. A phenomenon, called crosstalk exists between many phytohormones and has the meaning of crossing their signal pathways, so that the content of one phytohormone can influence the content or effect of the other. In the case of cytokinins, their content is strongly affected by ABA, not only endogenously, but after treatment with exogenous CKs as well (Pospisilova, 2003; Pospisilova et al., 2005). The decrease in the content of CKs in the cell also is a key factor for the induction of aging processes (Gan and Amasino, 1995). Cold stress and drought affect negatively the levels of CKs, as well. The change in their endogenous content after genetic transformation or exogenous treatment can also induce changes in the physiological activity of plants, which can affect their tolerance to stress. However, it must be considered, that the cytokinins are a large class of phytohormones and their action is very diverse and depends on the plant species, therefore the results from such experiments could be contradictory. For example, overexpression of genes, related to the degradation of CKs (cytokinin oxygenase, CKX) leads to growth of the root system, but is also related to decrease in the growth of the stem (Werner et al., 2003). The decrease in the CK levels using root-specific CKX genes is related to the increase of the root biomass. As a result, this genetic transformation gave the plants the ability for increased tolerance to drought. These genetically

modified plants had a higher chance for survival during unfavourable conditions, such as soil, low in nutrients or prolonged drought. On the other hand, the overexpression of the gene isopentenyl-transferase, *IPT* leads to a reduced growth of the roots and a decreased tolerance to drought. Tobacco plants, transformed with a gene for IPT, controlled by a promoter, inducing genes for protein kinases during senescence (SARK::*IPT*) demonstrated increased resistance to drought (Rivero et al., 2007). These transgenic plants showed a much better growth than the wild type after watering with 70% less water than the control conditions. The content of *trans*-zeatin was higher, as compared with the wild type. Furthermore, according to newer studies from the same authors, cytokinins induced the activation of photorespiration after drought in transformed plants (Rivero et al., 2009). Thus, cytokinins affect the process of photosynthesis, by protection against the formation of ROS.

Studies, using CKs to achieve tolerance to low temperatures, showed contradictory data. Exogenous treatment of bean plants /*Phaseolus vulgaris/* or sugar beet with cytokinins, as well as the increased CK levels in *IPT*-transgene *Festuca pratensis* increased the tolerance of these plants to cold stress (Veselova et al., 2005). On the other hand, the treatment with N6-benzyladenine of tree plants of Saskatoon berry /*Amelanchier alnifolia*/ significantly decreased their tolerance to cold stress. The decrease in the content of CK, induced after treatment with cold stress in wheat seedling is responsible for closing of the stomata. The treatment with exogenous N6-benzyladenine increased transpiration in plants, with roots subjected to cold stress, inducing a visible loss of turgor and wilting. Significant increase of the tolerance to low temperatures was observed in the highly sensitive to cold stress sugar cane (*Saccharum* sp.) after transformation with a gene for IPT, controlled by the cold-inducible promotor (AtCOR15a) (Belintani et al., 2012). This modification led to slowing down the aging process in leaves, increasing the efficiency of photosynthesis, postponing the offset of flowering and increasing the tolerance to unfavourable conditions. The increased content of endogenous CK was a reason for increasing the antioxidant activity (more efficiently quenching of ROS), which protects the photosynthetic apparatus during stress. This leads to increased tolerance to the stress factor and suppression of the programmed cell death. In addition, studies showed transgenic petunia and chrysanthemum, containing the *IPT*-gene controlled by the cold-inducible promoter COR15a from *Arabidopsis thaliana* (AtCOR15a::*IPT*) (normally controlling the gene for 15-kD cryoprotector peptide, specific for the chloroplasts). Results from these experiments displayed slow induction of aging after a period of acclimation to low temperature.

Regardless of the limited literature data that show the possible effects of CKs in the regulation of tolerance to cold stress, it gave us the reason to take a step to investigate the CK content in these three populations of *Arabis alpina*, after application of cold stress, as well as the transcription of key genes from their metabolism.

03. AIM AND TASKS

The aim of the current dissertation work was to study the regulation mechanisms of tolerance in *Arabis alpina* to cold stress. For this purpose we proposed the following particular experimental tasks:

1. Collection and identification of populations of *A. alpina* from the French Alps and Rila mountain, tolerant to minus temperatures (-7^{o}C).
2. Comparative analysis of tolerant and non-tolerant to low minus temperatures populations of *A. alpina*, as follows:

- measurement of electrolyte leakage from the plant cells to study their integrity, with the aim of determination different populations of *A. alpina*.
- determination the net photosynthetic activity using the device LCpro+
- determination the content of photosynthetic pigments (chlorophyll *a* and *b* and carotenoids) by the means of HPLC analysis.
- investigation the concentration of tocopherols as non-enzymatic markers for the antioxidant activity of the cells.

3. Determination of the functional state of PS II and PS I by the methods of the prompt chlorophyll fluorescence and the modulated 820 nm light reflection, respectively.
4. Determination of the endogenous content of phytohormones (ABA, cytokinins, jasmonic acid) by HPLS-GS analysis in the tolerant and non-tolerant to cold stress plant populations.
5. Investigation of the transcription rate (qRT-PCR analysis) of genes from the metabolism (ABA1; NCED3,5; AAO3; CYP707A1) and the signal transduction of ABA (ABCG40) and cytokinins (IPT1,2,3; CKX1,2,5,7 and ARR1,5,7), respectively.
6. Transcriptional (qRT-PCR) analysis of photosynthetic genes as follows:

- genes for proteins from PS II (PSBA; PSBH; PSBO2; OE23; PSBS)
- genes for proteins from PS I (PSAB; PSAD2; PSAG; PSAH1; PSAN; LHCAIII)
- other photosynthetic genes (PETA; PETC; RBCL; RBCS)

04. MATERIALS AND METHODS

04.01. Object of research, growth conditions and experimental design

- **Object of research: *Arabis alpina* L. (Brassicaceae)**

In our experimental work populations with different tolerance to negative temperatures of the plants *Arabis alpina* were used (Fig. 7). *Arabis alpina (Alpine rock-cress)* is a perennial, high mountain plant from the Brassicaceae family, a close relative species to the model plant *Arabidopsis thaliana* (Frantzke et al., 2010). Recently, the plant genome was sequences in the laboratory of prof. George Coupland from the university in Köln. The size of the plant genome is about 375 Mb (n = 8 chromosomes), the plant being a self-fertile diploid (Koch et al., 2008). Plant areals are located in the altitude from 500 to 3200 m, depending on the latitude. *Arabis alpina* can be found in all of Europe, North Africa and the Middle East. (Assenov et al., 1970; Rameau et al., 2008).

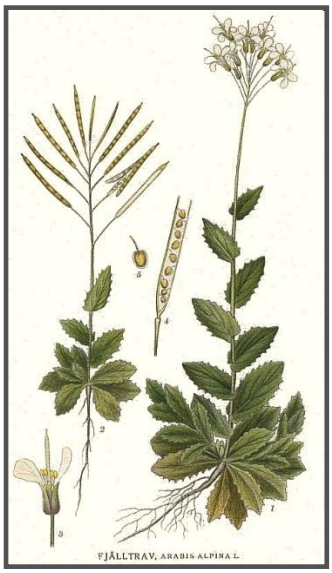


Fig. 7: Arabis alpina L.

For the purposes of the current work plant populations from the French Alps, as well as from Rila mountain were grown and treated in laboratory conditions. Seeds from three populations of *Arabis alpina* from the French Alps, pre-selected in the Laboratory of Alpine Ecology (LECA) on the basis of their tolerance to negative temperatures were kindly given to us from our French colleagues from the university "Joseph Fourier" in Grenoble, France. These populations included tolerant (T) from the passage of Galibier in the French Alps (2600 m alt.) and non-tolerant (NT) from the mountain of Vercors (1824 m alt.). Populations of *A. alpina* with short primary stem /hypocotyl/ (SH) were studied as well, which are moderately tolerant to freezing stress and possess a shortened stem part, as a phenotype for tolerance to snow (results from the study of the latter /SH/ plant population were not presented in the current work).

Fig. 8 Arabis alpina. A natural habitat in the area of the Seven Rila Lakes (the location between the lakes "Bliznaka" and "Trilistnika"), at the end of September 2013. The generative stem can be easily seen – tilt (in pale yellow colour) with the formed siliques, containing ripe seeds. Seeds from A. alpina were collected and introduced as soil cultures in laboratory conditions.

Fig. 9 Arabis alpina. Natural habitat in the area of the Seven Rila Lakes (the location between the lakes "Bliznaka" and "Trilistnika"), in the flowering period at the end of June 2014.

- **Methods for collecting of plant and seed samples**

Seeds from the fertile parts of the plants (siliques) from the French Alps were collected in the years 2009, 2010 and 2012 by the team of prof. Michel Herzog from the Laboratory of Alpine Ecology from the university in Grenoble. The collection was carried out by packing of plants, randomly chosen in the beginning of the flowering period with the aim to prevent the cross-pollination. Ten areals (4 in Vercors and 6 in Ecrins) were selected out on the basis of a previous study (Poncet et al., 2010), and two of them were chosen especially for this study, because plants there were strongly tolerant (GAL) and non-tolerant (F005) to freezing in standardised conditions (Table 1). We also studied wild populations of *A. alpina* from Rila mountain, found in the area of the Seven Rila lakes (between the lakes "Bliznaka" and "Trilistnika"; plants were growing as tuffs and most often they were hidden below juniper Fig. 8, 9). Plants from these tuffs were pricked out and transferred in laboratory conditions, as well as seeds were collected and introduced for their cultivation as soil cultures in laboratory conditions. In the cases of using whole plants from the nature, the tuff was extracted carefully from the soil, without affecting the individual plants, and afterwards every one of the individual plants were pricked out in a pot and cultivated in laboratory conditions. In the latter case the plants were not grown continuously, being developed well enough, but it was necessary to acclimate them at the new conditions (for about two weeks), after which it was possible for the treatment to be carried out, according to the experimental scheme (Table 3).

Table 1: Populations, differing by their tolerance to frost

Mountain	***area***	***altitude (m)***	***location***	***exposure***
Vercors	F005	1842 m	Côte2000II (Villard de Lans)	north
Ecrins	GAL	2600 m	Galibier	south-west
Рила	RILA	2300 m	"The Seven Lakes"	north-east

Table 2: a growth cycle of a culture form Arabis alpina in standartised laboratory conditions

	Stage	Duration (weeks)	Temperature (°C)	illumination ($\mu mol.m^{-2}.s^{-1}$)	photoperiod (h. day/night)
1	**Seed maturation**	**8**	**20-23**	**65**	**16**
2	**Seed collection**	**-**	**-**	**-**	**-**
3	**Dormancy**	**8-12**	**10**	**-**	**-**
4	**Stratification**	**1**	**4**	**-**	**-**
5	**Vegetative growth**	**8-20**	**20-23**	**65**	**16**
6	**Vernalisation**	**8-12**	**4**	**37**	**12**
7	**Flowering**	**8**	**20-23**	**65**	**16**

- **Plants, grown in standardised laboratory conditions, progeny of plants from the nature**

The seeds were gathered during autumn, when the siliques are already dry and were packed and stored at 10°C for a certain period, when it was necessary to be planted (Table 2). Five mature seeds were places inside pots, 8x8 cm wide, after placing 7 cm of moist substrate for cultures NFU 44-551 (containing a turf moss, plant compost and sand), the fertilisers used were NFU – 42001 (mineral and organic-mineral fertilisers with a composition of N, P, K 3 kg/m^3). The seeds were left for a week at temperature of 4 °C in the dark for stratification, to simulate the end of the winter period. Seeds were covered with a transparent film (mini-hothouse), during the first two weeks for the soil to be kept wet, to accelerate the germination and lower the mechanical vibrations. After that they were placed in a cultivation chamber for a period of 2-5 months in the following conditions: temperature 22°C±2 °C, photoperiod 16/8 h day/night, light intensity 220 μmol $m^{-2}s^{-1}$ and relative humidity 60-70%. After the first 10 days growth of the cotyledons was observed, and after about 15 days first true leaves appeared (Fig. 10). During the first month about 5-6 new true leaves appeared, but for the leaves to attain their normal size at least about two months were necessary. Plants were grown during at least two months and a half, until enough fully developed leaves formed. Flowering was observed after the phase of vernalisation that occurred for 8 to 12 weeks at 4°C and short photoperiod (12/12 h day/night) (Table 2). This phase is usually necessary for the perennial plants to activate the genes PEP1 and PEP2, responsible for the flowering (Bergonzi et al., 2013). Flowers appeared almost immediately after their removal from the low temperature chamber, and fertilizer with a content of NPK was added to avoid the loss of the siliques, that

often takes place in conditions of nutritional stress. The flowers were packed until ripening of the siliques, to prevent the cross-pollination.

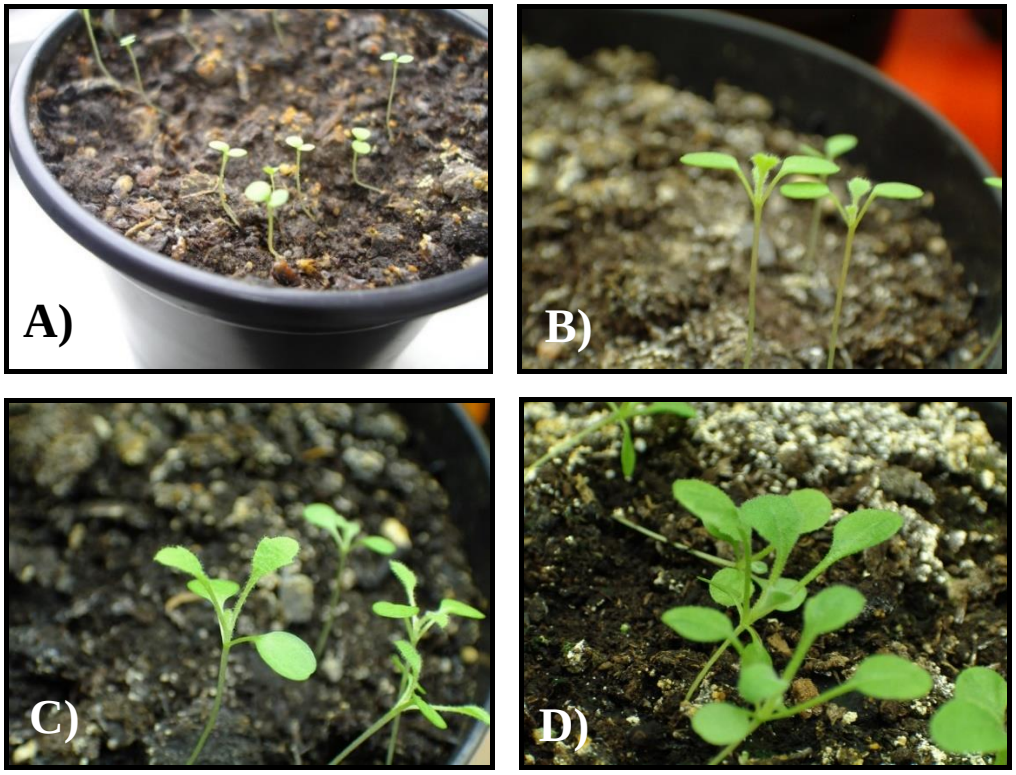


Fig. 10. A) 10-day, B) 15-day, C) 20-day, D) 30-day seedlings of Arabis alpina, from seeds, collected in the area of the Seven Rila Lakes and introduced in laboratory conditions as a soil culture.

- **Experimental design**

After plants were developed well enough or were acclimated in laboratory conditions (at temperature of about 22^{o}C), experimental treatment with low positive (*chilling stress*; at around 4^{o}C) and negative temperatures (*freezing stress*; at around -7^{o}C) could be carried out. Basis of each experiment was the setup from table 3, whereas some of the experiments were carried out without the recovery periods. Experimental setup consisted of exposure to chilling stress (4^{o}C) for 4 days, whereas other factors in the growth conditions (photoperiod, light intensity, relative humidity) remained unchanged. After that plants were treated in freezing conditions (-6-7^{o}C) as well, for 12 hours in the dark. Two periods of recovery followed, each consisting of 4 days. In the first recovery period plants were returned to the temperature of chilling (4^{o}C), and the last one was to return the plants at the temperature of cultivation

(22ºC), again for 4 days. Treatment with low positive and negative temperatures were conducted in a climatic chamber (TK 120, Nuve, Ankara, Turkey). Each measurement was carried out at room temperature, after exposure to the corresponding temperature of treatment according to the experimental scheme. The specific period of adaptation at physiological conditions, depending on the experimental setup was observed for each experiment. In the experiments, related to measurements of net photosynthesis by the means of CO_2 uptake and the stomatal conductivity, this period was about two hours, in the light at 22ºC. The adaptation for the measurements of chlorophyll fluorescence consisted of darkening the plants for about 20-30 min.

Table 3. Experimental setup of the treatment conditions of plants from the French populations of A. alpina (T and NT) and from the area of the Seven Rila Lakes.

Treatment **Populations**	**22°C Control**	**4°C; 4 days (chilling stress)**	**–7°C, 12h in the dark (freezing stress)**	**Recovery 4°C; 4 days**	**Recovery 22°C; 4 days**
Tolerant	**T_{22}**	**T_4**	**T_{4S}**	**Rec T_4**	**Rec T_{22}**
Non-tolerant	**NT_{22}**	**NT_4**	**NT_{4S}**	**Rec NT_4**	**Rec NT_{22}**
Rila	**$Rila_{22}$**	**$Rila_4$**	**$Rila_{4s}$**	**Rec $Rila_4$**	**Rec $Rila_{22}$**

04.02. Measurement of the electrolyte leakage from leaves, treated with different negative temperatures

When biological membranes are damaged, electrolytes from the cell interior together with water flow out of the cell. This process is called electrolyte leakage and is a consequence of disruption in the selective permeability of cell membranes. The measurement of the percentage of free electrolytes allows the stress damage upon the leaf to be evaluated. To simplify the model it could be assumed, that the part of the free electrolytes in the sample water is directly related to the level of leaf damage. The protocol for measurement is adapted from the method, developed by E. Azzarello in 2009.

For every experimental variant 4 individual plants were selected, passing a phase of pre-acclimation at 4ºC (Table 3). A leaf from each plant was taken in the base of the leaflet stem and a care was taken to protect the tissues from damage. The leaves were placed in 4 separate 2 ml Eppendorf ® test-tubes, containing 100 μL water UHQ (Ultra-pure quality, with conductivity $\sigma < 1$ μS); after that the samples were immediately cooled in a cryostat Julabo

F25-ED to 4 °C. The temperature was lowered at steps of 30 min each to -4 °C, -6 °C, -7 °C, -8 °C, -9 °C, -10 °C and -11 °C.

The four test-tubes were transferred on ice at the end of each of the corresponding phases at temperature of -7 °C, -9 °C, -10 °C and -11 °C. Afterwards the leaflets were transferred at room temperature in autoclavable test-tubes, with a content of 20 mL UHQ water and were paced on a shaker for 15 hours at 160 rpm. 2 to 6 repeats from a sample were carried out. The control contained only 20 mL UHQ water, without leaf sample and was placed for mixing in the same way. The conductivity was measured in every test-tube (measurement 1), then the value of the control (only with water) was subtracted from the obtained results. The tubes were autoclaved for 20 min on 121°C for determination of the maximal conductivity (100% of electrolyte leakage) after cooling.

The ratio of the electrolytes, released from the leaf after and before autoclaving is equal to the proportion of the cell damage during stress. LT_{50} (semi lethal temperature) is a threshold value for the temperature tolerance of the population and is obtained graphically, when the electrolyte leakage is 50%. The lower this threshold, the more tolerant to freezing is the plant from the current population. Leaves with different age from a plant were studied, every time after a different period of acclimation after 4 ^{o}C, for the optimization of protocol.

04.03. Measurement of the net photosynthetic activity by the method of CO_2 uptake

Net photosynthetic activity was also measured, by the means of LCpro+ (ADC BioScientific Ltd., UK). It was used to determine the net photosynthesis on the basis of the uptake of CO_2 from the plant. This device has LEDs, emitting 500 μmol photons $m^{-2}s^{-1}$ PAR (photosynthetically active radiation) of saturating light, and can maintain a stationary background concentration of CO_2 (by using a constant current of CO_2), as well. Three tolerant plants (T), three non-tolerant (NT) and three plants from Rila mountain were used in the measurements. They were carried out after every treatment with stress temperature (*chilling* at 4^{o}C, *freezing* at -6^{o}C), as well as the control temperature at 22^{o}C and the recovery after stress at 4^{o}C and 22^{o}C. Plants were left for adaptation for two hours at the conditions of the control (22^{o}C, 220 μmol photons $m^{-2}s^{-1}$) before measurements. The desired effect, aimed by this procedure was the opening of plant stomata, and distinguishing the real photosynthetic activity (the capturing of CO_2 by Rubisco) from the transpiration. From two to four leaves from each plant were used for at least three measurements on a leaf. Taking into consideration the very small area of the leaflets from *Arabis alpina*, the narrow leaf chamber of the device

(5.80 x 1.00 cm) was used. Before placement of the leaves inside the chamber, the area of every leaf was measured, and the corresponding settings of the device were made, to reflect the real area of the leaf.

04.04. Measurement of the chlorophyll fluorescence with the PEA and M-PEA fluorometers

We used the method of the prompt chlorophyll fluorescence (PF) as an instrument for *in vivo* analysis of the condition of the photosynthetic apparatus. The measurements were performed with the two devices from the firm Hansatech, UK, the PEA (Plant Efficiency Analyser), which gives information about the prompt chlorophyll fluorescence (PF) and the M-PEA (Multifunctional Plant Efficiency Analyser) can give much more detailed information for the PF, and also carries out measurements by two more methods for analysis of the photosynthetic activity, namely the delayed fluorescence (DF) and the modulated 820 nm light reflection (MR_{820}).

The prompt chlorophyll fluorescence of the chlorophyll *a* (PF) and the modulated 820 nm light reflection (MR_{820}) were measured simultaneously with the help of the multi-functional analyser of the plant photosynthetic activity, the M-PEA (Multifunctional Plant Efficiency Analyser; Hansatech Instruments Ltd., King's Lynn, Norfolk, PE30 4NE, UK). The apparatus measures the kinetics of the so-called delayed fluorescence of the chlorophyll *a* (DF) as well, but the results were not described in the current work. M-PEA consists of a sensor unit for the measurements and a control block to record, analyse and convert the results in electronic variant and, finally, to send them to a computer. The setup of the M-PEA sensor unit consists of three emitter and four detector devices. The LEDs for the actinic light emit light with wavelength in the range of 627± 10 nm, the LEDs for the modulated light emit at 820± 25 nm, and the wavelength of the emitters for the far-red light is about 735± 15 nm. The latter use a filter with a transmittance in the spectrum of the far-red light (RG9), to eliminate an eventual component from the visible light. High-quality optical lens filters are included in the construction of the device for protection of the detectors for PF and DF (730± 15 nm) and the MR_{820} (820± 20 nm). The light emitting diode, radiating in the range of the far-red light spectrum (735±15 nm; 1000 μmol photons $m^{-2}s^{-1}$ at 100%), can be used when a fast recovery in the redox condition of the reaction centres is necessary (e.g. from light-adapted samples). In addition, the measurement of the reflection of this light beam, in a combination with measurements from wavelengths 627, 735 and 820 nm (by the fourth, wide-spectrum

detector) allows determination of relative absorption of the leaf (it was not used in the current study).

LEDs for the actinic light are mounted in the centre of the optical sensor element and are focused at the surface of the sample, for a homogenous lightening to be secured upon an open circular area (4 mm in a diameter) with the intensity of the light flux up to 5000 μmol photons per $m^{-2}s^{-1}$ (in this current work light intensity of 4000 μmol photons per $m^{-2}s^{-1}$ was applied). (Strasser et al., 2010)

For measurement of the fluorescence dark adapted (for 20-30 min at room temperature), well developed intact middle-aged leaves from *A. alpina* plant were used. The measurements were carried out at room temperature after exposure of the plants to low temperatures with different intensities (*chilling* and *freezing*: 4ºC and -6ºC, respectively) and the control temperature (22ºC), as well as the recovery after freezing at 4ºC and 22ºC, respectively. Leave samples were placed in a clip with a circular aperture in the middle (4 mm in a diameter), which is a part from the M-PEA apparatus with a screen for the aperture to be kept closed during the dark adaptation. During measurements, carried out by the device a light beam in the red wavelength range with a duration of 10 sec (for the PF measurements) and photon flux intensity of 4000 μmol photons per $m^{-2}s^{-1}$ was applied. The emitted by the plant fluorescence was recorded by a PIN photodiode detector with an optical line filter, passing the light with wavelength 730 nm. Measurements of the MR820 were carried out by applying actinic light with 627 nm and a modulated, optically filtered pulse with 820 nm. The signal was recorded by a PIN photodetector diode with an optical lens filter in the range of 820 nm. Three independent and reproducible experiments were carried out, each with different plants *A. alpina* from T and NT populations from the Alps, as well as with plants (including plants, grown from seeds) from the Rila mountain. During every single experiment at least 3 samples of leaves from a plant were measured and the results were statistically analysed.

Except graphs (JIP curves, Kautsky curves), drawn on the basis of the fluorescence of leaf samples from *A. alpina*, parameters of the fluorescence were obtained, as well. They were calculated from the values of fluorescence in the characteristic phases of the fluorescence kinetics, the so-called OJIP stages from the transient curve. These phases are denoted differently in the literature, depending on the methods and apparats used for the study of chlorophyll fluorescence. Two of the points from the transient OJIP curve that are used most often in the literature, are denoted as F_O-initial fluorescence, when all reaction centres are open, i.e. they are in the oxidized state and F_M-maximal fluorescence when all reaction

centres are closed (reduced, i.e. in an excited state). Most devices give F_O as the value, measured in the very start of the fluorescence (at the first 10-50 milliseconds), and the F_M is the value of the maximal fluorescence (matching the F_P, or the fluorescence at 300 ms when illuminating normal, non-stressed plants with saturating light). These parameters correspond to the points O and P from the transient curve, respectively. Other important phases from the transient curve of the fluorescence are J and I, at 2 and 30 ms, respectively. They reflect the fluorescence, produced after reduction of Q_A from the PS II (the fluorescence at J is denoted as F_J), and the reduction of the PQ, b_6f complex and the final electron acceptors of PS I (fluorescence at I is denoted as F_I), respectively. Some authors denote the phase K, which can be observed in stress conditions before the point J (F_K). The values of the fluorescence, obtained in the different phases at the progress of the transient curve, can be presented as relations to each other. As a result, so-called parameters of the fluorescence are calculated. They are a measure of the energy fluxes, passing inside the PS II.

Analysis of the parameters of prompt chlorophyll fluorescence

The value of the so-called variable fluorescence is given by the difference between the maximal (when all reaction centres are closed; F_M), and the initial fluorescence (when all the reaction centres are open; F_O), and is equal to the trapped energy flux ($TR_O = F_V = F_M - F_O$). The ratio of this variable fluorescence (F_V) to F_M ($TR_O/ABS = \varphi_{Po} = F_V/F_M$) represents the levels of the fluorescence when maximal amount of the reaction centres of PS II are open, or the so-called quantum yield of the primary photochemical reaction (the reduction of the RC of PS II and the phaeophytin).

The difference between the maximal F_M and the fluorescence in phase J (2 ms) gives the ET – electron transport flux, ($ET_O = F_M – F_J$); the ratio of which with the F_M is $ET_O/ABS = \varphi_{Eo} = F_M – F_J / F_M$ and describes the quantum yield of the electron transport after Q_A.

RE – Reduction of end electron acceptors at PS I acceptor side, ($RE_O = F_M – F_I$; $RE_O/ABS = \varphi_{Ro} = F_M – F_I / F_M$) is the ratio of the difference between the maximal F_M and the fluorescence in phase I (30 ms). This is the parameter, describing the quantum yield of electron transport at the reduction of the end electron acceptors from the side of PS I.

Other very important parameter is ψ, ($ET_O/TR_O = F_M – F_J / F_M - F_O$; $ET_O/TR = \psi_o = F_M – F_J /F_V$), the ratio of the differences between F_M and F_J and the variable fluorescence reflecting the probability for the movement of an electron beyond Q_A. Furthermore, PI, or the

performance index is used as well, representing the overall state of the photosynthetic apparatus. It is a much more complex parameter and includes the product of several other simpler parameters, such as the φ quantum yields. In addition, other parameters exist, with a lower application in practice.

04.05. Determination of the quantity of the photosynthetic pigments and phytohormones by the means of HPLC

Pigments were extracted from lyophilised samples by methanol (neutralized with 5mM Tris-HCl, pH 7.0) The samples were separated by HPLC on a C30 column with a reverse phase (250x4.6 mm), manufactured by YMC Co. Ltd and bought by Interchim (France). Mobile phases used were: methanol (A), water/methanol (20/80 volume parts), containing 0.2% ammonium acetate (B) and tert-methyl butyl ether (C). Gradient used were 95% A / 5% B during 12 min, a step to 80% A / 5% B and 15% C for 12 min, followed by a linear gradient until 30% A / 5% B / 65% C for 30 min. The eluent profiles were analysed by absorption detector with a diode array. The quantitative determination was achieved by the means of curves, sensitive to the containment, and the identification of carotenoids was achieved by comparing the spectral properties of standards. Detection of tocopherols was completed by the means of the same procedure with a fluorescent detector.

Extraction and determination of the phytohormone content

Endogenic phytohormones were extracted from 100-200 mg fresh weight in methanol/formic acid/water (15/1/4, v/v/v) and purified by a dual-mode solid-phase extraction method, which allows the separation of the hormones on basic and acidic by consequential eluting with Oasis MCX column (Waters Co, Milford, MA, USA). The extraction of phytohormones was completed using the method of Dobrev and Kaminek, 2002 that separates the phytohormone extract in two fractions: 1) fraction A, containing acidic and neutral phytohormones and 2) fraction B contained basic hormones. Hormones were determined quantitatively by HPLC (Ultimate 3000, Dionex), coupled to a triple-quadruple / linear ion trap-masspectrometer (3200 Q TRAP, Applied Biosystems). The first fraction contained the acidic hormones (ABA, Jasmonic acid), whereas the second fraction contained the basic hormones (cytokinins).

04.06. Isolation of total cell RNA and qRT-PCR transcription analysis of genes for proteins from the photosynthetic apparatus and for enzymes from the metabolism and signal transduction of phytohormones

qRT-PCR analysis is a method for quantitative determination of the transcription of genes by the means of reverse transcription of mRNA and then PCR amplification of the obtained cDNA fragments and their detection with dye. qRT-PCR is a highly-sensitive method allowing for the analysis of rare transcripts and very small changes in the gene expression (Pfaffl, 2001). For the simplest technique for detection of newly-synthesised PCR products in RT-PCR is used the fluorescent dye SYBR Green I, that binds specifically to the small groove of the double-stranded DNA. The quantitative method for detection depends on the target sequence, the quantity of mRNA expected in the sample, the needed level of accuracy and whether it is necessary for the quantitative determination to be absolute or relative. The latter represents a comparison between the transcription of genes of interest and a reference gene (most often house-keeping genes like genes for tubulins, actins, albumins, 18S rRNA or 28S rRNA are used for such purpose). Absolute determination is based on an internal or external calibration curve.

qRT-PCR analysis was carried out in France, Grenoble, Laboratory of Alpine Ecology (LECA) in collaboration with our French colleagues. Experimental set-up of treatment with low temperatures was standardised between Bulgaria and France (see table 3). mRNA extraction was completed immediately after the night period. The total RNA was extracted from stem leaves by incubation with TRIzol® Reagent (Invitrogen) and with the help of RNeasy Plant Mini Kit (Qiagen). Briefly, 100 mg leaves from *A. alpina* were grinded in a liquid nitrogen and transferred in 1 mL Trizol (and after that stirred on vortex at room temperature for about 5 min). 300 μL of chloroform were added (mixing for 15 sec) and were centrifuged at 12 000 g for 10 min at 4°C. Next, the water phase was separated in a new test-tube and 0.53 volume of absolute ethanol was added. The solution was transferred in RNeasy column (Qiagen kit). The following steps were described in the protocol of Qiagen. The genome DNA was removed using RNase-free DNase Kit (Qiagen) according to the protocol of the manufacturer. After that the total RNA aliquot was analysed with a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Depending on the extract, for the synthesis of the first sequence of cDNA 1–2 μg total RNA and SuperScript™ III Reverse Transcriptase Kit (Invitrogen) were used after annealing with non-specific hexamer primers. Finally, cDNA concentration was determined and was diluted to appropriate volume.

qRT-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) with a final reaction volume of 25 μL (12,5 μL master mix, 5 μL cDNA, 7,5 μL primer mix) by the apparatus MyIQ RT-PCR, BioRad. Melting temperature of the DNA fragments was about 60ºC. A PCR cycle comprised of 15 sec denaturation at 95ºC and 30 sec renaturation at 55ºC. In the beginning and the end of the renaturation process, obtained fragments were recorded by the device, after detection with SYBR Green. Each sample was analysed as a triplicate within the frames of a single experiment and the relative transcriptional levels were calculated based on the reference gene for 18S rRNA (*18sRNA = At1g13320*). PCR triplicates were carried out with three separate cDNAs, each of them corresponding to the RNA extracted from a tissue of at least three plants. During the data analysis, results from wells, giving abnormal amplification graphs or double melting curves were eliminated. The data for qRT-PCR was analysed according to the comparative method using the formula C_t ($2^{-\Delta\Delta Ct}$), where ΔC_T was calculated for every sample as the difference between C_t of the studied gene and C_t of the reference gene (e.g. 18S rRNA). Fragments of cDNA, about 20bp long were used as primers, and sequences, around 80-150bp long were used for DNA amplification. Smaller sequences were not enough reliable statistically, whereas longer ones had a higher probability to warp around and anneal during replication. Primer design was completed by the means of the software for design of primers "Primer 3", and the data for the sequence of the cDNA was obtained from the database of Arabidopsis TAIR. Primers, corresponding for the genes from *A. alpina* were obtained with the kindly co-operation of prof. George Coupland from the University of „Max-Planck", Koln. These primer sequences, used for amplification are shown in table 4.

Table of the primers used in the qRT-PCR analysis

Arabis alpina / Arabidopsis ID	***Abbrev.***	***Gene***	***Function***	***Sequence (5'->3')***	
AT3G41768	*18SRNA*	18S Ribosomal RNA	18S ribosomal RNA, encoded in the nucleus	**F**	GCATTTGCCAAGGATGTTTTC
				R	GCGGAGTCCTATAAGCAAC
AT5G67030	*ABA1*	Aba Deficient 1	Codes a single copy of the zeaxanthin-epoxidase gene, functioning on the first step from the ABA biosynthesis.	**F**	AGCTGCCTGAAGTAATTTACCA
				R	GCGGCGTTTAAGGTGAAAGT
AaNCED3	*NCED3*	Nine-cis-epoxycaro-tenoid di-oxygenase	Codes for the isoform 3 of the gene for the enzy-me 9-cis-epoxycaroten-oid dioxygenase	**F**	TGTTAACCCCAAAGCCAAAG
				R	GTGTTGTCTCTCGTGGCTGA
AaNCED5	*NCED5*	Nine-cis-epoxycaro-tenoid di-oxygenase	Codes for the isoform 5 of the gene for the enzy-me 9-cis-epoxycaroten-oid dioxygenase	**F**	ACGGCGCTAATCCACTCTT
				R	AACTCGCGTCTCCGTTATTG
AT2G27150	*AAO3*	Abscisic Aldehyde Oxidase 3	Coding for the delta iso-form of the enzyme alde-hyde oxidase, catalysing the last step from the biosynthesis of ABA.	**F**	TTGCATTGCCAGATGAAGAC
				R	CAGCAATTACCGCATGTACG
AaCYP707A1	*CYP707A1*	Cyto-chrome P450	Coding for the enzyme cytochrome P450, oxi-dizing ABA to 9 or 8 OH-ABA.	**F**	TCAAGTCCAGAAGCAGCAAA
				R	CCTTGGTGGAAAAAGATTGC
AaABCG40	*ABCG40*	ATP-bind-ing casset-te (ABC) transporter	Coding for transport protein from the signal transduction of ABA	**F**	CCATGCAGGGTAGAAGAGGA
				R	CTCTCTACACGGGAGCCTTG
AaIPT1	*IPT1*	Isopenteny ladenine transferase	Coding for the gene for synthesis of biologically active cytokinins (iso-pentenyladenine trans-ferase)	**F**	CGATAAGGCGGTGGATGATA
				R	CAACTCTCTTAATCTCCCAACCTG
AaIPT2	*IPT 2*	Isopenteny ladenine transferase	Coding for the gene for synthesis of cis-zeatins (from tRNA)	**F**	GGAAAATCTGAGGAAGGTACTGG
				R	CCTTCTGGTATTTAACTTCACCCTATC
AaIPT3	*IPT3*	Isopenteny ladenine transferase	Coding for the gene from the multigene family of isopentenyladenine transferase	**F**	TAGCACCCATGATGACCACA
				R	CCTCCTTCTCCGGCTTTAGA

ATCKX1	*CKX1*	Cytokinin oxidase/dehydrogenase	Coding for a gene from the multigene family of the CK oxidase, degrading the cytokinins with unsaturated double bond in the isoprene chain	**F**	CTTTGGCAAAAGATGGGAAA
				R	GCGAGTTGGATGGGAGATAA
AaCKX2	*CKX2*	Cytokinin oxidase/dehydrogenase	Coding for a gene from the multigene family of the CK oxidase	**F**	TCTGGGTTCCACCTCAAATC
				R	TGTTGATGACAACACCTTGATG
AaCKX5	*CKX5*	Cytokinin oxidase/dehydrogenase	Coding for a gene from the multigene family of the CK oxidase	**F**	TTCATCTCAACCACCACACC
				R	CAACCATCCTCAACCGAAG
AaCKX7	*CKX7*	Cytokinin oxidase/dehydrogenase	Coding for a gene from the multigene family of the CK oxidase	**F**	GGAGCTGGCTGTAGCAAAAC
				R	AGTTGTAACGGGAAATGGTGA
AaARR1	*ARR1*	Arabidopsis response regulator	Coding for a transcription factor from the signal transduction of the cytokinins (isoform 1)	**F**	AGATGAGGAAGGGGAAGAGC
				R	AGCGACAAACTGCTGATGC
AaARR5	*ARR5*	Arabidopsis response regulator	Coding for a transcription factor from the signal transduction of the cytokinins (isoform 5)	**F**	TTGAAGCCTGTGAAATTAGCTG
				R	ATCTTTGCGCGTTTTAGCTG
AaARR7	*ARR7*	Arabidopsis response regulator	Coding for a transcription factor from the signal transduction of the cytokinins (isoform 7)	**F**	CGGTGAGGTTATGAGGATGG
				R	TATCATCGACGGCAAGAACA
AT2G27150	*AAO3*	Abscisic Aldehyde Oxidase 3	Coding for the delta isoform of the ABA aldehyde oxidase	**F**	TTGCATTGCCAGATGAAGAC
				R	CAGCAATTACCGCATGTACG
AT5G67030	*ABA1*	Aba Deficient 1	Coding for a single copy of the gene for the zeaxanthin-epoxydase	**F**	AGCTGCCTGAAGTAATTTACCA
				R	GCGGCGTTTAAGGTGAAAGT
AT5G23120	HCF136	high chlorophyll fluorescence 136	Coding for a factor of the stability and structure of PS II	**F**	GGCTCCCATGTCAAAAAGAA
				R	GGAACCTTCAGTGCGGTTAAT
AT1G61520	LHCA-III	Photosystem I Light Harvesting Complex Gene 3	PS I-type III chlorophyll a/b binding protein (Lhca3*1) (antennae protein)	**F**	TGAGGCTGGTCAAGACATTG
				R	GCTATGCTCGCTATCCTTGG

AT1G06680	OE23	Photosystem II Subunit P	Coding for 23 kD protein from PS II acting in the 0_2 formation. Its phosphorylation is Ca^{2+} dependent.	**F**	TGCTATGCTTGCTTTCTTCATT
				R	AGCGAAGCCGAAAAGGTAAC
AT4G22240	PAP	Plastid-lipid associated protein	Protein from the gene family of the fibrillin (structural)	**F**	GCACAAAGACACTTCCACCA
				R	AACGATATCGAGCCAACCAC
ATCG00540	PETA	Photo-synthetic Electron Transfer A	Coding for the cytochrome f apoprotein; involved in the photosynthetic electron transport; coded by the chloroplast genome; its transcription is inhibited from the nuclear gene HCF2.	**F**	AAAAATTTGTGCCAAAACAACC
				R	AGGGGGATGCGGAAATAGTA
AT4G03280	PETC	Photo-synthetic Electron Transfer C	Coding for the FeS Rieske center from the cytochrome b6f complex	**F**	TTGTTTGTCAAGCGACGAGT
				R	GGAGGGGCAAAGAAAGTAGC
ATCG00340	PSAB	Photosystem I Subunit D-1	Coding for the D1 subunit from the reaction centre of PS II	**F**	TAAACTTTGGGTTTGTGGATGGA
				R	TTTATGCTCAAAACCCCGATTC
AT1G03130	PSAD-2	Photosystem I Subunit D-2	Coding for the protein sequence, similar to PsaD from spinach, a protein subunit from the reaction centre of PS I (PsaD2)	**F**	CCGCAGCTAGACCCTAACAC
				R	TCCTGTTGGCATCTCAAAGA
AT1G55670	PSAG	Photosystem I Subunit G	Coding for the G subunit from PS I, 11-kDa membrane protein, acting in the electron transport between PC and PS I and stabilises PS I	**F**	CGAAACAGGGCTTACCAGAA
				R	CCCAAGCAAGAACATCAACA
AT3G16140	PSAH1	Photosystem I Subunit H-1	Coding for the VI subunit H from the reaction centre of PS II	**F**	TGGAGGAGGCTCTTTGCTTA
				R	ACCGAGCTTAGGTGGCTCTT
AT3G50820	PSBO2	Photosystem II Subunit O-2	Coding for the protein from the content of PS II, involved mainly in the stabilizing the manganese cluster	**F**	AGTTGGCCAAGGAAAATGTG
				R	CCGAAGGCTGAATACTCTCG

AT1G44575	PSBS	Photo-system II Subunit S	Coding for the PS II-S (CP22), a pigment bound protein associated to PS II in higher plants	**F**	AGTGGCTCGATCTCGTTGAT
				R	ATTGGCACAGCTGGGAATAG
AT3G46780	PTAC16	Plastid Transcrip-tionally Active 16	Chloroplast transcription factor	**F**	GCAAGGCGAAAGATATTGGA
				R	GCCTTTGCTTGTCCTCTGAC
ATCG00490	RBCL	Large Subunit Of Rubisco	Coding for the big subunit of RUBISCO. The protein is a tyrosine-phosphorylated and its phosphorylation condition is modulated as a response to ABA	**F**	TTACAAAGGACGATGCTACCAC
				R	TGAACCCAAATACATTACCCACAA
AT5G38430	RBCS	Ribulose Bisphos-phate Car-boxylase Small Chain 1A	Coding for the protein from the multigene family of the small Rubisco subunit (RBCS). Functions to supply enough amount of Rubisco for the photosynthetic activity of the leaf	**F**	ATCACTTCCATCGCAAGCA
				R	CCACTTGTTGCGGATAAGGTA

Table 4 Table of the studied by the means of qRT-PCR genes and their function (by TAIR) and the sequence of the primers, allowing their identification.

04.07. Statistical analysis of the results

Statistical analysis of the experimental data was performed with the software packages Excel and SigmaStat. Statistical significance of the differences between different experimental variants was assessed by the variance analysis (ANOVA one-way analysis) at P=0.05. Statistically significant differences between the experimental variants were represented on the graphs as small Latin letters. The mean value of the results was presented for every experimental variant, together with its standard error.

05. RESULTS AND DISCUSSION

05.01. Effect of the low temperatures on plants from T and NT populations from the Alps, as well as on plants from Rila mountain

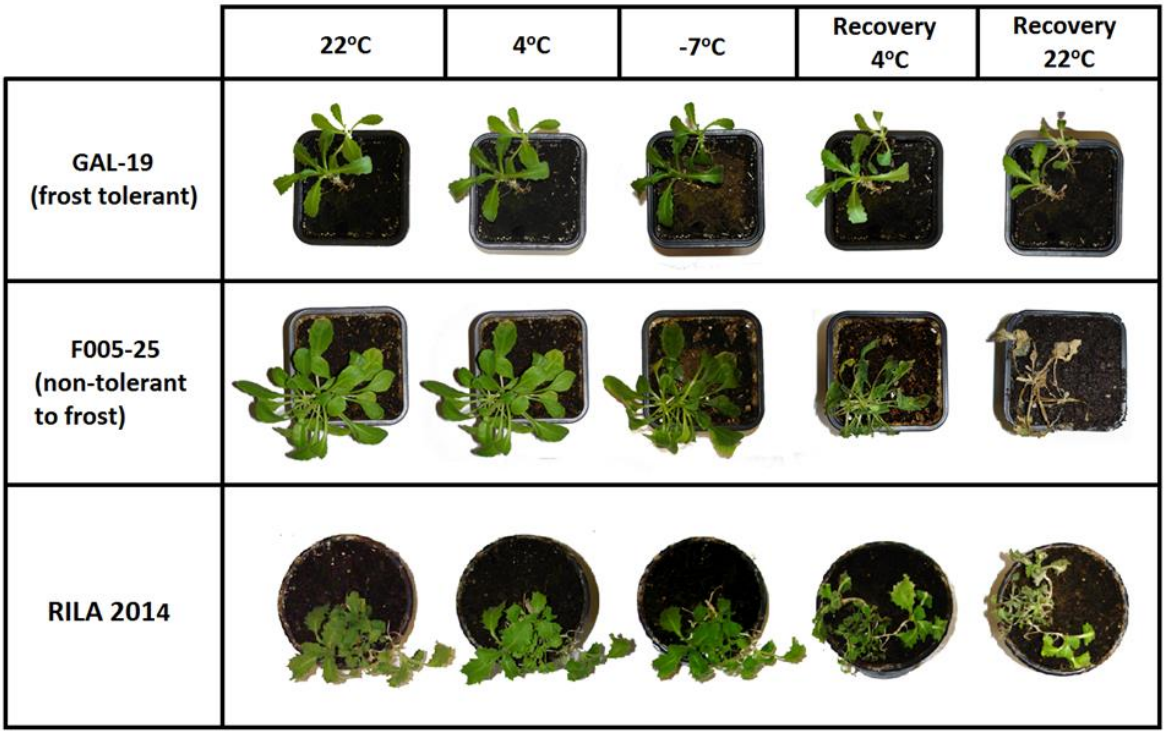


Fig. 11 Effect of the cold stress on plants of Arabis alpina, from the populations GAL-T (tolerant) and F005-25 (non-tolerant), as well as from Rila mountain (RILA 2014). Plants were grown at 22°C and exposed subsequently at chilling (4°C) and freezing stresses (-7°C), as well as to recovery subsequently at 4°C and 22°C according to the experimental scheme (Table 3). The experiment was carried out with 12/12h photoperiod, 70% rel. humidity and light intensity of 220 µmol m^{-2} s^{-1}.

Plants from three populations of *A. alpina* were collected and a phenotypical analysis was conducted on them, based on their tolerance to minus temperatures. The first population from the French Alps, the passway of Col du Galibier (2600 m altitude) turned out to be a tolerant to the low minus temperatures (-7°C). Plants from this population were denoted as GAL / T (tolerant). The next studied population from the Vercors mountain (1800 m altitude) was non-tolerant to freezing stress (-7°C). These plants were denoted as F005 / NT (non-tolerant). The last population of *A. alpina* was picked out from the Rila mountain, the area of the "Seven Rila lakes" (2300 m altitude) denoted with RILA. The plants from this population were highly tolerant to low minus temperatures. Mature seeds from the three populations were

collected, and all of the experiments were carried out with plants from seeds, grown in laboratory conditions.

Plants from the two French populations (GAL-19 and F005-25) and the populations from Rila mountain were subjected subsequently to low temperature (*chilling*) stress at 4ºC and freezing stress at -7ºC, as well as to two periods of recovery at 4ºC and 22ºC (Fig. 11). The three populations of *A. alpina* were tolerant to the temperature 4ºC, as can be seen from the Fig. 11, however, they differ by their tolerance, concerning temperatures below 0ºC. At temperature of -7ºC plants from the population GAL-19 and plants from Rila mountain did not show significant changes in their phenotypes. In contrast to them, plants from the F005-25 population lost their turgor of the leaf lamina, representing the characteristic change in phenotype after low temperature stress (Fig. 11). The damage exerted on the plants from non-tolerant population (F005-25) increased after both periods of recovery and finally most of the plants died. After the frost stress, some of the plants from Rila mountain showed damage to parts of their leaves (Fig. 11), as well and this was clearly visible after the periods of recovery.

05.02. Measurement of the electrolyte leakage

Electrolyte leakage from plant cells is a direct indicator for the viability of the cell as a function of the integrity in the membrane system of the cell. In case the membrane and cell wall were not intact (their integrity was disrupted somehow), the leakage of electrolytes should be higher. In this way the borders of plant tolerance to stress could be determined very precisely. Fig. 12 shows that plants from non-tolerant population had a significant level of electrolyte leakage, around and higher than 80% even at -7ºC, whereas plants from tolerant population presented electrolyte leakage below 10% at the temperatures of -7ºC, -8ºC and -9ºC, and only at -10 ºC it was increased up to 80%.

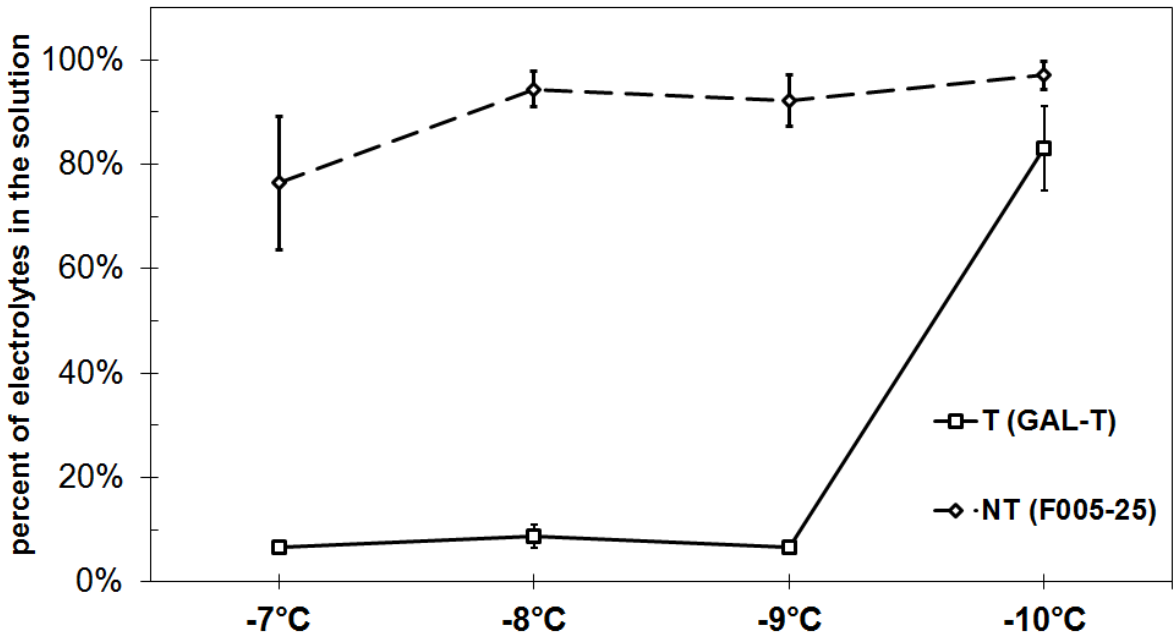


Fig. 12 A percentage ratio of the electrolytes freed in plants from populations of Arabis alpina (T), (NT). Leaves from the plants were placed in double distilled water and were treated at different temperatures (-7°C, -8°C, -9°C and -10 °C). The amount of freed electrolytes was determined as a percent from the control.

05.03. Content of chlorophyll *a* and *b* and carotenoid pigments

- **Content of chlorophyll pigments**

Changes in the content of chlorophyll *a* and *b* under the effect of the different temperatures is represented on Fig. 13. During the treatment with low temperatures a gradual decrease in the content of both types of chlorophyll was observed, and this decrease was more pronounced in the content of chlorophyll *a* in both low temperatures. After the chilling stress (4°C), the decrease in the content of chlorophyll *a* and *b* in plants from tolerant population T was 13% and 18%, respectively. After treatment with -7°C chlorophyll *a* was decreased almost two-fold (24%) in the same population, whereas the chlorophyll *b* remained almost unchanged (20%). In the non-tolerant to freezing stress population NT, the same tendency of a stronger decrease in chlorophyll *a* was observed gradually, after treatment with 4°C (24% inhibition) and -7°C (40% inhibition). The content of the chlorophyll *b* in the NT population was also decreased, but to a much less extent (18% after chilling, and 34% after -7°C, Fig. 13).

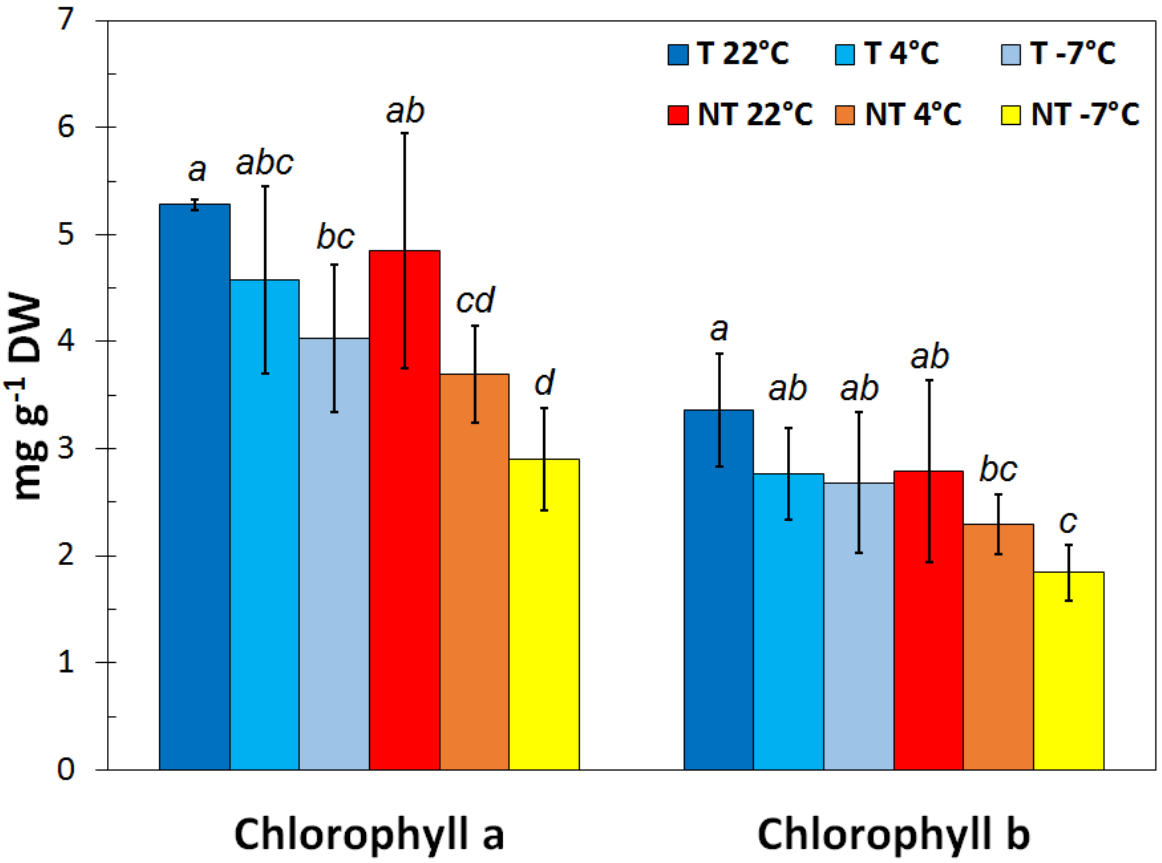


Fig. 13 Content of chlorophyll a and b in tolerant (T) and non-tolerant (NT) to freezing stress populations of Arabis alpina, in conditions of cold stress (chilling 4°C and freezing stress -7°C). Small letters show the statistically significant differences at P = 0.05.

The changes in the content of chlorophyll pigments after treatment with low temperatures were typical for abiotic stress (stress-induced aging, for example light stress, nitrogen deficiency), where initially the content of chlorophyll *a* decreases much more strongly, as compared with the chlorophyll *b*, like in the case of normal aging process (Kura-Hotta et al., 1987; Hidema et al., 1992). These changes in the chlorophyll content are related to the degradation of a greater part from the reaction centres of both photosystems, in contrast to the antennae complexes (Humbeck et al., 1996; Miersch et al., 2000).

- **Content of carotenoid pigments**

According to the HPLC analysis of the carotenoid pigments in the leaves of *A. alpina*, the content of lutein was the highest (end product from the metabolic chain of α-carotene) as well as that of β-carotene (Fig. 14), the lutein content being two times higher than the β-carotene. The content of the xanthophyll pigments (zeaxanthin, violaxanthin, neoxanthin) was significantly lower, and the content of the antheraxanthin was negligible and was not presented on the graph (Fig. 14). After the periods of subsequent chilling (4°C) and freezing

(-7°C) of the plants, the total carotenoid content did not change almost at all in the T population, whereas in NT population it was sharply decreased (28% after 4°C and 34% after -7°C) (Fig. 15). In contrast to the T population, where the content of both main carotenoids lutein and β-carotene was maintained almost unchanged after the decrease of the temperature to 4 °C, in the NT population it was strongly decreased with about 20-25%, and this decrease was even more clearly visible for the lutein (30-35%). This stronger decrease of the content of carotenoid pigments in NT was observed for the pigment from the xanthophyll cycle *trans*-violaxanthin, as well. The behaviour of the second xanthophyll pigment zeaxanthin, which is extremely important in stress conditions, displayed increased after the cold stress, and mostly after the treatment with -7°C.

The content of lutein is in highest amount from all carotenoid pigments in higher plants, and in both studied populations of *A. alpina* it was about 54 - 57% from the total pigment concentration (Fig. 14). The next carotenoid pigment by percentage in higher plants is the β-carotene (Bungard et al., 1997). Its relative part in *A. alpina* was between 23-25%. Neoxanthin in *A. alpina* was represented between 3-4% and did not change in both studied populations after the subsequent treatments with different temperatures according to the experimental scheme (22°C, 4°C and -7°C). The content of neoxanthin usually does not change in higher plants, as compared with the content of other carotenoid pigments (Johnson et al., 1993; Bungard et al., 1997). In the case of *A. alpina* its content remained unchanged in plants from both populations after applying the cold stress. Finally, as a last representative of the carotenoid pigments, the 9-cis-violaxanthin also did not change significantly after the cold stress, and its content was in between 8-9%.

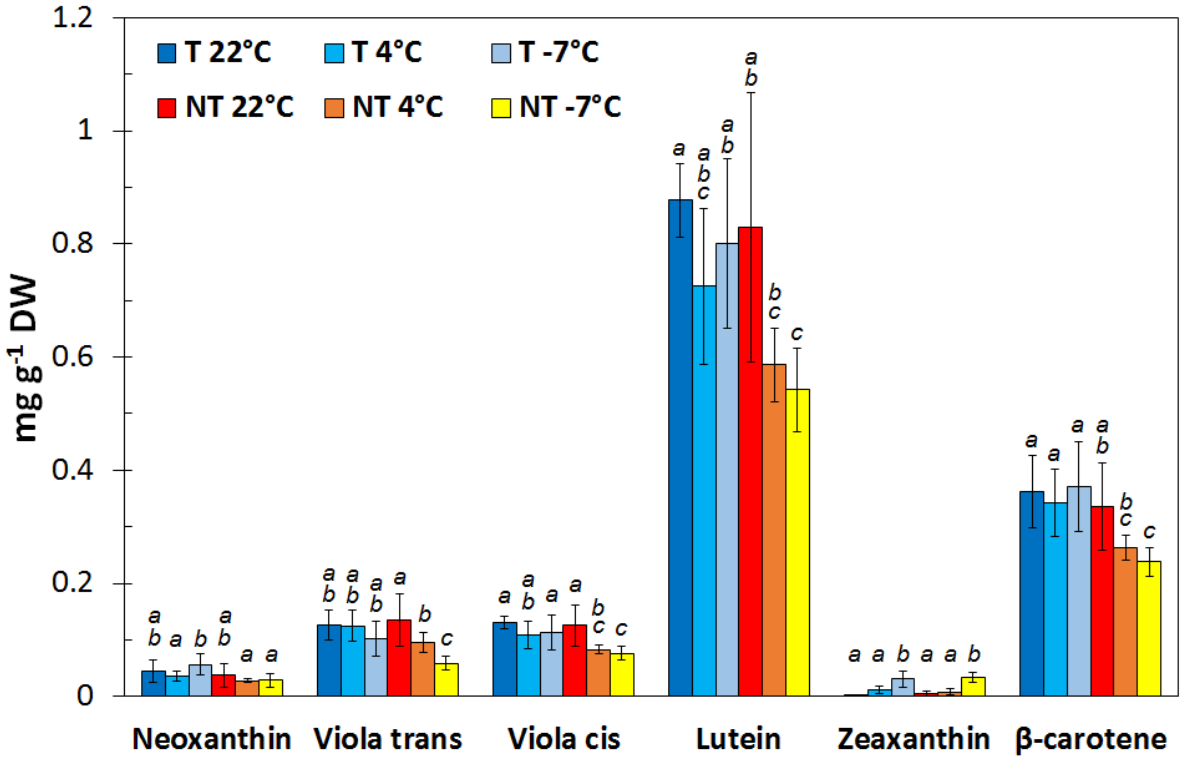


Fig. 14 *Content of carotenoid pigments in T and NT populations of Arabis alpina, in conditions of cold stress (chilling, +4°C and freezing stress, -7°C). Small letters show statistically significant differences at P = 0.05.*

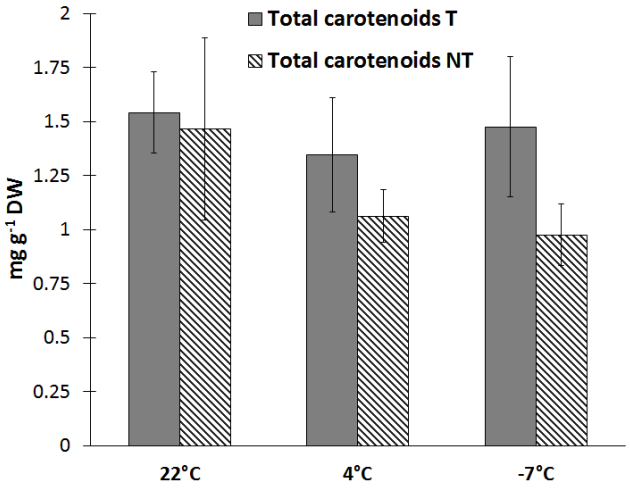


Fig. 15 *Total content of carotenoid pigments in T and NT populations of Arabis alpina, in conditions of cold stress (chilling stress, +4°C and freezing stress, -7°C).*

Concerning the carotenoid pigments from the xanthophyll cycle (zeaxanthin and *trans*-violaxanthin), their content was affected mostly after the cold stress (Fig. 14, 16). In the control variants of both populations, zeaxanthin was represented in very low concentrations, but its content was increased significantly in T and NT after -7°C, reaching close values in both populations (Fig. 14). The *trans*-violaxanthin, widely known to de-epoxidise to zeaxanthin after conditions of stress (Niyogi et al., 1998), was decreased after the minus stress on the account of rising in zeaxanthin concentrations (Fig. 16). The results, represented as a ratio between the zeaxanthin and the total amount of the pigments from the xanthophyll cycle were interesting and were shown on Fig. 16. It can be clearly seen, that in T, as well as NT the ratio gradually changes in the favour of zeaxanthin after the subsequent treatments with low temperatures.

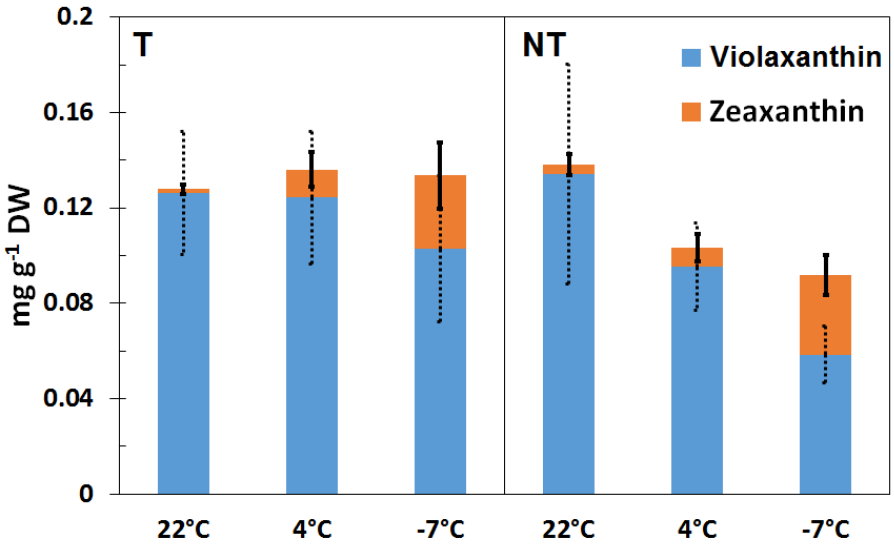


Fig. 16 *The ratio between the content of zeaxanthin and trans-violaxanthin (the two main pigments from the xanthophyll cycle) in T and NT populations from Arabis alpina, in conditions of cold stress (chilling, +4°C and freezing, -7°C).*

The content of carotenoid pigments in *A. alpina*, described by us in the former section, is comparable with the literature data for these pigments in *Arabidopsis thaliana*, as well as in other plant species (Bungard et al., 1999; Verhoeven et al., 1999; Havaux et al., 2007). Changes in the content of main carotenoid pigments lutein and β-carotene were observed mostly in the NT population after treatment with 4°C and -7°C, can be interpreted as an effect of the stress in this population, as compared to T population, where change was not measured (Fig. 14, 16). On the other hand the change in the content of both pigments from the xanthophyll cycle (decrease of *trans*-violaxanthin and increase of zeaxanthin) under the effect

of the low temperatures (Fig. 14, 16) in both populations of *A. alpina* showed the activation of the xanthophyll cycle. These results confirm the role of zeaxanthin in the reactions for neutralising the effect of the cold temperatures in *Arabis alpina*. It must be emphasised, that exactly after -7^{o}C the content of zeaxanthin in both populations reached its highest content during the whole experiment. Concerning the third pigment from the xanthophyll cycle (antheraxanthin), its insignificant concentration (that is not presented on the graphs) is most probably due to its transition state in the cycle, although according to some studies its content increased after stress, as well (Verhoeven et al., 1999).

Many literature data showed that the activation of xanthophyll cycle is a protection mechanism against photodamage, exerted usually at high intensity of light in combination with abiotic stress (Sarry et al., 1994; Havaux et al., 2000). It is well-known, that the involvement of the zeaxanthin as a photoprotector in plants relates mostly to the non-photochemical quenching of the excess energy from the photosynthesis (Niyogi et al., 1998). Furthermore, zeaxanthin could exert protecting action against the photodamage by other means, as well; for example as a stabiliser of the thylakoid membranes (Gruszecki and Strzalka, 2004) and as a quencher of the reactive oxygen species (Havaux and Niyogi, 1999; Johnson et al., 2007). According to newer literature data, the xanthophyll cycle could be activated after abiotic stress even in absence of light (Fernandez-Marin et al., 2011). Thus, xanthophyll cycle is an important and fundamental part from the response of the plant to different stress factors, mostly intensive light, but also high, low temperatures, drought and other stresses. It must be noted, that the increased content of zeaxanthin in *A. alpina* was observed in plants from the T population, as well as in NT plants, which did not survive after -7^{o}C. The overall content of pigments from the xanthophyll cycle, however, remained constant in T, whereas in NT it decreased strongly even after 4^{o}C, similarly to the rest of the carotenoid pigments (Fig. 16). Therefore, the activation of xanthophyll cycle could not be a reason for the tolerance to low temperatures in T, but this was due to the maintaining of concentration of the carotenoid pigments in plants from T population, in contrast to NT. The dynamics in the overall amount of the pigments from xanthophyll cycle was very close to the total carotenoid pigments (Fig. 14, 16). Because the concentration of the carotenoid pigments in T remained constant, and decreased in NT, the activation of the xanthophyll cycle in NT could not compensate for the overall decrease in the physiological activity as a result from the cold stress.

As a conclusion, the zeaxanthin formed after the activation of the xanthophyll cycle was involved in the protection against the cold stress and especially freezing in *A. alpina*. In the first place, it is an important mean for protection against excess light (photoprotection), as well as against the ROS released after the abiotic stress. Moreover, it protects the thylakoid membranes against oxidation in conditions of abiotic stresses. All of this led to the conclusion, that the carotenoid pigments, especially the lutein, β-carotene as structural pigments and the zeaxanthin as a photoprotector are important elements from the tolerance to low temperatures in *A. alpina*.

- **Content of tocopherol antioxidants**

Parallel with the content of chlorophyll and carotenoid pigments in *A. alpina*, the concentration of different forms of tocopherol (vit. E) was determined, being one of the fundamental components of the antioxidant system in plants, as well as in animals. In stress conditions the photosynthetic apparatus is stabilised mainly by the antioxidant systems (Cheng and Ma, 2004). In both plant populations of *A. alpina* the content of α-tocopherol was much higher (15 μM g^{-1} DW or about 80%), as compared with the rest of tocopherol species (Fig. 17). With the decrease of the temperature, the concentration of α-tocopherol remained unchanged in T, as well as in NT. The amount of β-tocopherol was about six times lower than that of α-tocopherol and also remained constant in both plant populations and stress temperatures. The content of the rest two tocopherols (γ and δ) were lower than the β-tocopherol, and the content of δ-tocopherol was the lowest from the all other forms of tocopherol. In contrast to α- and β-tocopherol, the content of γ- and δ- tocopherols was increased only in plants from the tolerant population after subsequent treatment with low temperatures.

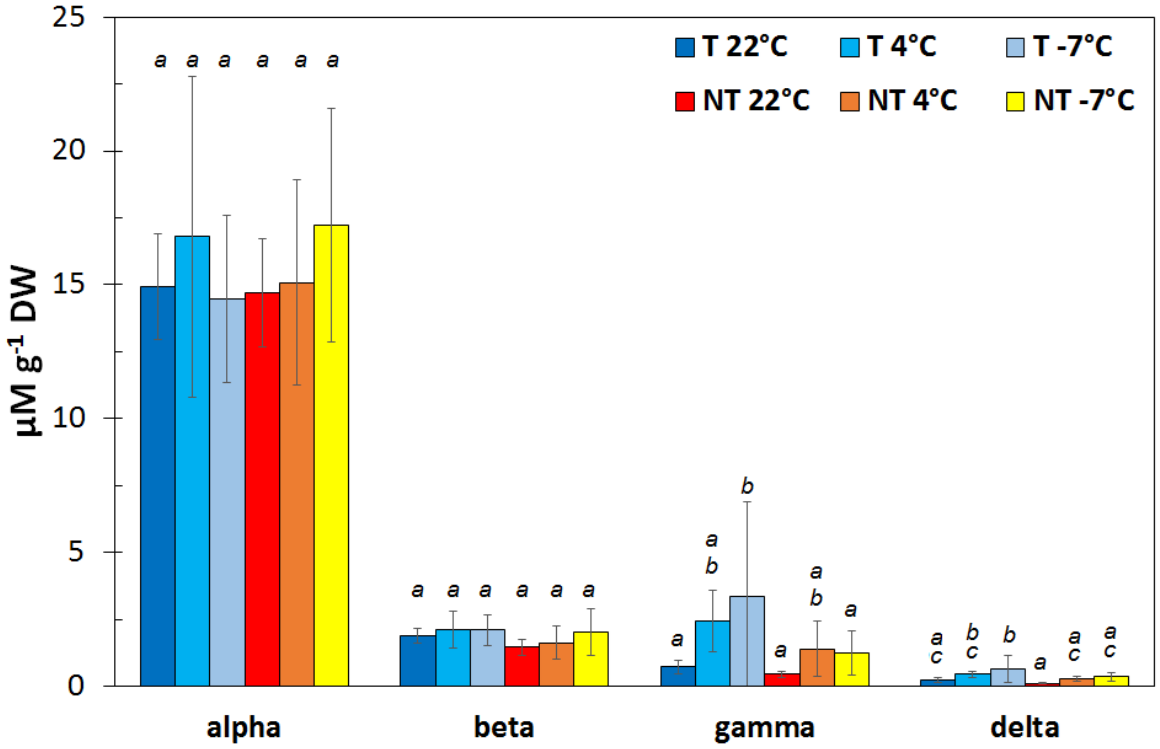


Fig. 17 Tocopherol content in T and NT populations from Arabis alpina, in conditions of cold stress (chilling, +4°C and freezing stress, -7°C). Small letters indicate the statistically significant differences at P = 0.05.

The results from the percentage amount of the four types of tocopherols to their total amount (Fig. 18) showed unchanged content of the main form of tocopherol, the α-tocopherol, as well as the β-tocopherol in both plant populations of *A. alpina.* As written in the former paragraph, the only exception was the content of γ-tocopherol and to a lesser extent the δ-tocopherol, with increase after the cold stress, especially after -7°C. This increase in the two types of tocopherol was much strongly revealed in the population T (3-4 fold) as compared with the NT population (about 2 fold).

It is well-known, that the tocopherols, among which is the vital for a large amount of organisms (plants, animals, microorganisms) vitamin E (α-tocopherol), are extremely strong antioxidants. One of the most important functions of α-tocopherol is the function of a strong antioxidant, i.e. a quencher of reactive oxygen species (ROS). In plants, the tocopherols along with the carotenoids act in the protection of the photosynthetic apparatus against damage from different types of abiotic stress (Szarka et al, 2012). Furthermore, tocopherols stabilise the cell and mostly the thylakoid membranes, preventing the lipid peroxidation (Havaux et al., 2003). In addition, tocopherols could be involved in regulation of the biosynthesis of jasmonates, the

latter known as the cell mediators of stress (DellaPena, 2003; Munné-Bosch, 2005). Their function of protection of the cell against ROS is tightly bound to deactivation of the singlet

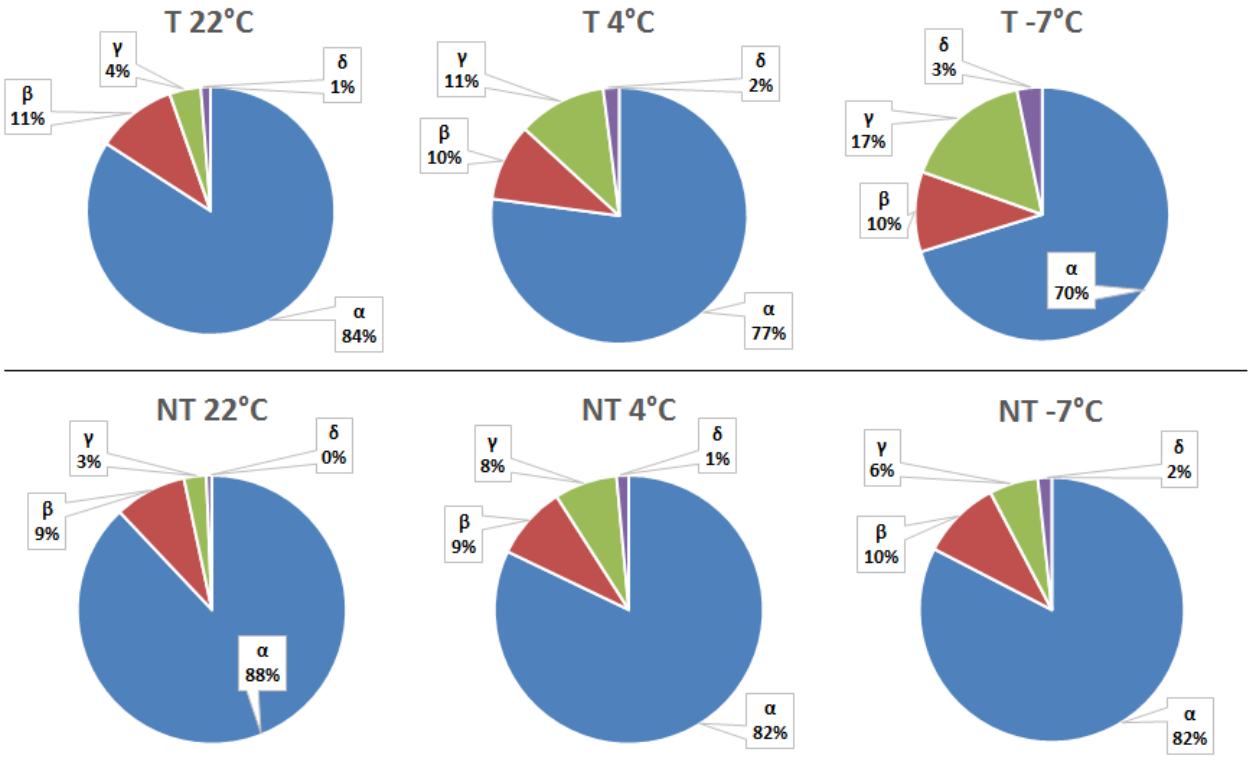


Fig. 18 Percentage content of α-, β-, γ- and δ- *tocopherol in different populations of Arabis alpina after cold stress. The amount of tocopherol was determined in leaves of plants from the populations T and NT of A. alpina after treatment with low temperatures (chilling stress, +4°C and freezing stress, -7°C, see Materials and Methods).*

oxygen forms, as well as the quenching of the excess light energy during photosynthesis (photoprotection) (Kruk et al., 2005). This is exceptionally important for the function of PS II, where in the reaction centre under strong light triplet forms of chlorophyll could be generated from P680. In the absence of tocopherol (or other deactivators of ROS), the singlet oxygen, formed by the triplet chlorophyll activates the degradation of the core protein D_1 from the reaction centre of PS II, P680 (Long et al., 1994; Melis, 1999). It should be noted, however, that in the other core protein of PS II, D_2, where β-carotene exists in the composition of this protein as a photoprotector, in D_1 such photoprotector is absent. As deactivators of the reactive oxygen species, tocopherols are considered the direct factors in the acquisition of plant tolerance against the photodamage from excess light energy, combined with abiotic stress (Kruk et al., 2005). Thus, tocopherols are the main factor that is responsible for the

tolerance of PS II to the effects of abiotic stress factors, and more importantly against photodamage, that inevitably accompanies most types of stress.

In the current dissertation work, α-tocopherol, which is the main bioactive tocopherol in most organisms, known as vit. E was in the highest amount in both studied populations (Fig. 18). It should be noted, that its content did not change after both types of cold stress. Only the γ-tocopherol showed significant increase after freezing in T, whereas in NT its content was not changed. According to some studies γ-tocopherol has similar, and even stronger protective effect than α-tocopherol, regarding the prevention of abiotic stress (Abbasi et al., 2007; Kruk et al., 2005). Tobacco plants (*Nicotiana tabacum*), containing mutant form of the gene γ-TMT, coding for the synthesis of the α-tocopherol from γ-tocopherol showed better response to oxidative stress (Abbasi et al., 2007). In these mutant plants, in contrast to the wild type, instead of the α-tocopherol, γ-tocopherol was accumulated. According to the authors of the same study (Abbasi et al., 2007), even the different types of osmotic stress (NaCl, sorbitol), exerted different effect between the plants from mutant and wild type. γ-tocopherol is a direct precursor in the metabolism of the α-tocopherol and it is possible in *A. alpina* to have mutual impact in their biosynthesis after the cold stress. It is interesting, that the content of α- and β-tocopherol remained constant in both populations after stress. Most probably, this is indicative for the dynamics in the synthesis and degradation of tocopherols during stress. β-tocopherol is produced from δ-tocopherol by methylation and for its synthesis the same enzyme γ-TMT is responsible, which also produces α-tocopherol by methylation the hydroquinone ring of γ-tocopherol. The content of δ-tocopherol was also increased after stress mainly in the T population (2 times higher than NT after -7^{o}C, Fig. 18), but nevertheless its content was the lowest compared with the rest tocopherols. The low values of the δ-tocopherol concentration show, that most probably it could not be an important factor in acquisition of tolerance to the cold stress.

05.04. Net photosynthetic activity

The net photosynthetic activity, as measured by the uptake of CO_2, is mostly a measurement for the activity of the Calvin-Benson cycle and the reactions from the dark phase of the photosynthesis. The results from the measurement of the photosynthetic activity of plants from the French populations T and NT showed a significant decrease after the first treatment with chilling stress (4 ^{o}C) (Fig. 19). At this temperature inhibition of photosynthesis with 41% in plants from T population and even higher inhibition in plants from NT

population (64%) was observed. After treatment with freezing stress (-7ºC), inhibition of the photosynthesis in T increased to 63%, and NT to 86%. The results from experiments with plants, grown from seeds, collected from the area of the "Seven Rila Lakes" showed, that changes in the net photosynthetic activity follow the same levels of inhibition, such as the French population of tolerant plants T (Fig.19). Inhibition of photosynthesis in the Bulgarian individual plants of *A. alpina* at 4ºC and -7ºC was 57% and 77%, respectively. It is very important to note, that from the plants placed in a recovery regime only the plants from populations T and those from the "Seven Rila Lakes" were able to recover their photosynthetic activity with a subsequent increase at 4ºC and 22ºC, respectively, after the treatment with frost stress. Individual plants from the population NT were not able to overcome the treatment with low temperatures and did not survive. Based on these results a conclusion could be made, that the populations of *A. alpina* from Rila showed a high level of tolerance to the negative temperatures, similarly to the T-population from the Alps. It is well-known, that photosynthesis is relatively high efficient process in its phase of the thylakoid reactions (light phase), but in the phase of the carbon reaction (dark phase) this efficiency decreased significantly (Taiz and Zeiger, 2006). This tendency was shown in our results, as well. It is considered, that the low activity of the fixation of CO_2 (e.g. in conditions of cold stress) affects negatively metabolism of the plant and leads to accumulation of high amount of reduced compounds, such as NADPH, which itself could be a reason for generation of ROS (Szarka et al., 2012). Plants with high levels of fixation of CO_2, have higher metabolic activity, but the accumulation of reduced compounds, and thus reactive oxygen species is lower. Furthermore, literature data show that it is possible for Rubisco of the high mountain plants to retain its activity, but as an oxygenase, thus switching to photorespiration to capture the excess oxygen and to prevent ROS formation (Manuel et al., 1999).

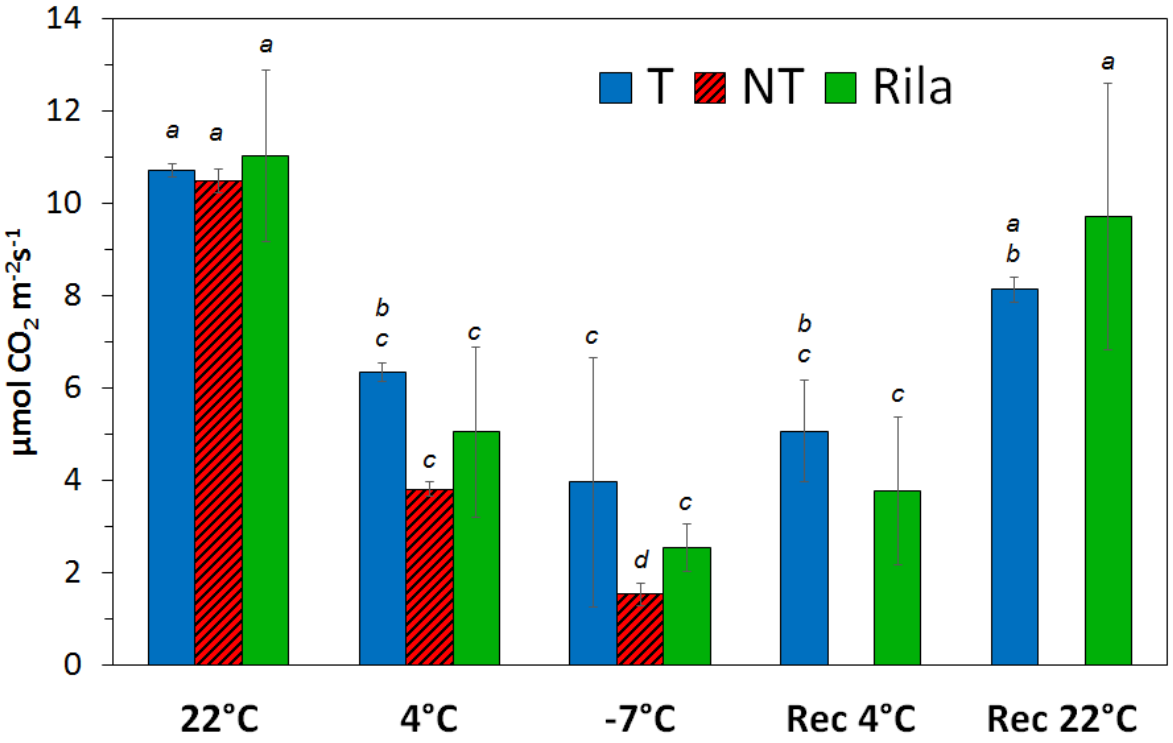


Fig. 19 Levels of net photosynthesis in populations of Arabis alpina (T, GAL-T), (NT, F005-25) from France and from the area of the Seven Rila Lakes (Rila). Plants were subsequently treated with different temperatures (22°C, 4°C and -7 °C) according to the experimental scheme and after a period of recovery at 4°C and 22°C. The activity of the photosynthesis was measured with LCpro+ by the method of the uptake of CO_2. Small letters indicate statistically significant differences at P = 0.05.

05.05. Studying the physiological condition of PS II and PS I by the means of the chlorophyll *a* fluorescence and modulated 820 nm light reflection

- **prompt chlorophyll *a* fluorescence**

As a measure of the activity of both photosystems (PS II and PS I respectively), methods of the prompt chlorophyll *a* fluorescence (PF) and the modulated 820 nm light reflection (MR_{820}) were used. Chlorophyll fluorescence is an indicator for the intensity of the reactions from the light phase of photosynthesis, and thus for the structural and functional condition of the photosynthetic apparatus. PF gives a detailed information for structural and functional state of PS II and indirect information for the state of the photosynthetic apparatus after it (Strasser et al., 2010). It is known, that PF is based on the emittion of the reaction centres of PS II after illumination with actinic red light (around 650 nm) (Goltsev et al., 2010;

Goltsev et al., 2014). In our experiments, the kinetics of the PF was measured with the fluorometers PEA and M-PEA, both of them allowing for the chlorophyll fluorescence to be registered, by the so-called transient JIP-curves, but M-PEA is also able to measure the reflection of far-red light from PS I (MR_{820}), thus PS I activity. Except that, M-PEA is able for simultaneously measurement of both signals (PF and MR_{820}) (Goltsev et al., 2010).

The chlorophyll fluorescence of dark adapted leaves (for about 30 min) of *A. alpina*, was measured after treatment with low positive and negative temperatures, according to the experimental scheme (see Materials and Methods). Three independent physiological experiments were carried out (in June 2013, November 2013 and February 2014), each one of them with 5 separate plants, and 3 leaves from the middle parts of the plant *A. alpina* from the French populations T and NT, respectively (Fig. 20, Fig. 22, Fig. 24), as well as with intact wild plants and plants, grown from seeds from Rila mountain (Fig. 21, 23, 25). Averaged results from these three experiments were represented on the forementioned figures.

The observed increase in the chlorophyll fluorescence after illumination with a duration of a second is described by the so-called JIP-curve. This curve characterizes with several characteristic points: O, J, I and P being most important of them (Strasser et al., 1995; 1997; 2004). All of these points represent a quasi-stationary state in the fluorescence emittion and correspond mainly to the subsequent events from the capture of a quantum of light energy from the PS II, transport of electrons on the oxidizing-reducing chain to the end electron acceptors of PS I and their reduction (Strasser et al., 2004). In the start of the fluorescent measurement (p. O), most of the P680 are open, thus the fluorescence is minimal (F_O), whereas in p. P fluorescence is maximal (F_P), because most of the P680-centres exist in a closed state. The points J and I reflect the accumulation of the reduced forms of Q_A and PQ, respectively.

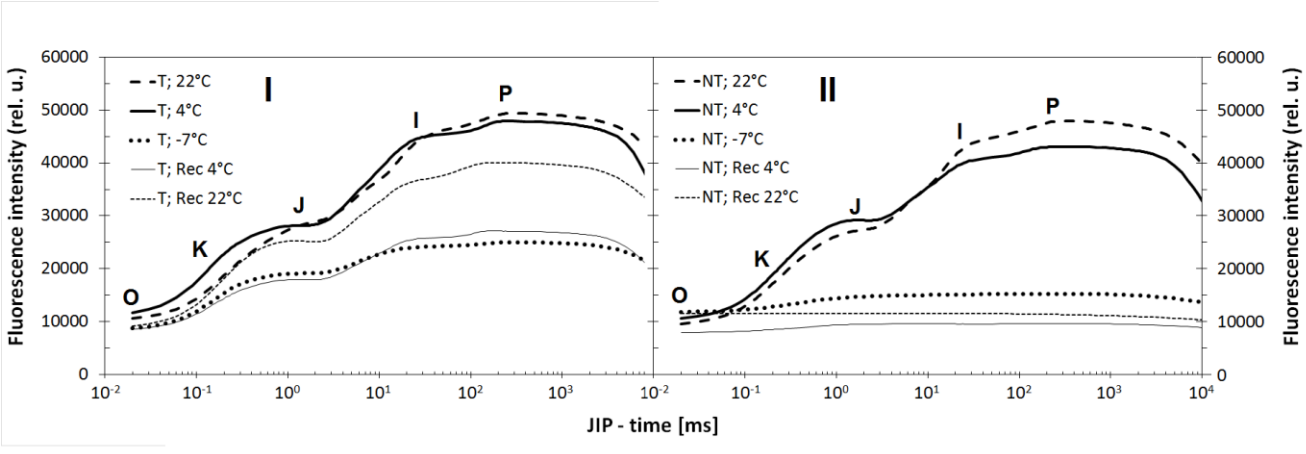


Fig. 20 Induction curves of the prompt chlorophyll fluorescence (PF) as a measure for the activity of PS II. The measurement was carried out with individual plants from the tolerant (T) and non-tolerant (NT) populations of Arabis alpina, in conditions of cold stress (chilling +4°C and freezing stress -7°C). The transient fluorescent JIP–curves were registered with the M-PEA (Hansatech, UK) device at 650 nm after dark adaptation (30 min) and intensity of the illumination 4000 µmol m^{-2} s^{-1}

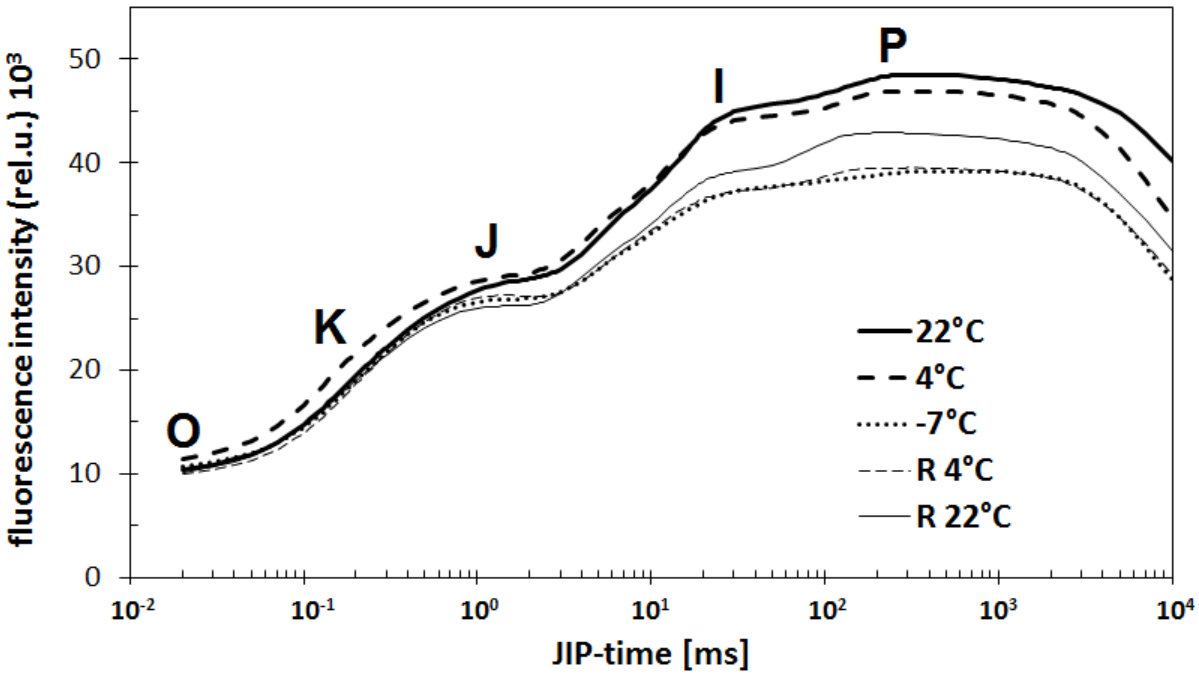


Fig. 21 Induction JIP-curve of the prompt chlorophyll fluorescence in leaves from A. alpina, grown in a climatic from seeds, collected in Rila mountain. Plants were exposed to chilling (4°C) and freezing (-7 °C) stress and afterwards to a recovery at 4°C and 22°C (see materials and methods) (R – recovery).

At the control temperature of 22°C, the T and NT populations of *A. alpina* (Fig. 20), as well as the plants of the population from Rila mountain (Fig. 21) showed identical transient curves, containing all typical characteristics of the classical fluorescent OJIP curve. After treatment with chilling temperature (4°C), the maximal fluorescence (F_M) in both populations was decreased. In non-tolerant plants (NT) the decrease was 10%, and in the tolerant (T) it was only 2%. It is well-known, that normally after stress the fluorescence decreases (Goltsev et al., 2014). The most characteristic change of the transient curve after 4°C in the T plants from France was the faster reaching of the J pike (the movement of the exponential part of the curve before p. J on the left) (Fig. 20, T). Changes of the transient curve in plants from Rila after 4°C showed strong similarity with the T (Fig. 21). The pike J was also moved towards

left (it was attained earlier), and the maximal fluorescence was decreased slightly. In contrast to T, in NT was observed increase of the intensity of the fluorescence in the phase J (Fig. 20, NT), which was also shown in a study of *Arabidopsis* at low temperatures (Nellaepalli et al., 2012). After the applied cold stress at 4ºC increase in the initial values of the fluorescence (F_O) was also observed in the plants from the three populations (T, NT and Rila), that was measured by other authors after cold stress in *Arabidopsis* (Mishra et al., 2011, Nellaepalli et al., 2012), as well as after drought in *Haberlea rhodopensis* (Strasser et al., 2010). A greater increase in the initial fluorescence was seen after -7ºC in plants from the NT population, as well.

After freezing the change in the induction curves in T and especially in NT was significant (Fig. 20). In T population after -7ºC the values of F_M were decreased two fold as compared with 22ºC (25 000 rel.u. at -7ºC compared to 50 000 rel.u. at 22ºC), in contrast to NT, where after the period of freezing it was further decreased with almost 40% as compared with T after -7ºC (25 000 rel.u. at T compared to 15 000 rel.u. at NT). The sharp decrease of the maximal fluorescence in NT was accompanied with the disappearance of the phase I-P. Although the activity of the photosynthesis and mostly PS II was decreased by a half in T after freezing, the normal shape of the curve of functional photosynthetic apparatus was preserved in the studied plants from this population. The separate phases O-J, J-I and I-P were clearly distinguishable, although strongly inhibited, as well as the intensity points of the fluorescence F_J, F_I and F_P. In contrast to T, where the characteristic phases of the transient curve O-J-I-P remained clearly visible in the conditions of freezing, in NT such retain was not observed, but on the contrary, the curve was compromised (Fig. 20, NT). After the phase J, the next stages were merged and was not possible to be determined. In the plants from Rila changes in the proceeding of the transient curve were observed, similarly to T. The values of F_M and F_J were additionally inhibited after -7ºC, but to a lesser extent, as compared with T. The separate phases were also preserved, but the amplitude of the phase I-P was decreased, and the point P was not at its usual place of 300 ms on the abscissa.

In the subsequent recovery period after the applied freezing stress, at temperature 4ºC and 22ºC, respectively, these differences in the appearance and behaviour of T and NT populations were increased and became much larger. The tolerant plants T gradually returned the normal motion of their transient O-J-I-P curves, whereas in the non-tolerant plants the normal OJIP appearance was lost and the curve degraded (Fig. 21). The slow recovery of the stages from the light phase of the photosynthesis was noticeable, and it was expressed by the

transient curves of PF and mainly by F_M. After the recovery at 4°C, the fluorescence was increased as compared with -7°C, but remained lower than the first treatment at 4°C. After the second period of recovery at 22°C the intensity of the fluorescence was increased further, but still below the values of the control or even the first treatment with 4°C. In the plants from Rila mountain was also observed such slow increase, similar to the T population after the periods of recovery (Fig. 22). The phase I-P, as well as the point P in 300 ms however, were recovered even after the first return of the temperature to 4°C, and the amplitude of the phase I-P was restored to its control values after the return at 22°C (Fig. 22).

At the control temperature 22°C all studied populations of *A. alpina*, including the French T and NT, as well as the Bulgarian plants from Rila mountain showed identical transient curves, containing all typical characteristics of the classical fluorescent OJIP curve, the points O, J, I and P were well-developed and after the expected millisecond intervals. After the staying of the plants for 4 days at temperature of 4°C (chilling stress) difference started to appear in the behaviour of the photosynthetic apparatus of plants from the three studied populations, namely the two French populations T and NT and the population from Rila mountain. Usually, this decrease in the maximal fluorescence (F_M) is typical for stress conditions (Strauss et al., 2007; Strasser et al. 2010, Mishra et al., 2011, Venkatesh et al., 2012). It is mostly due to the degradation of the proteins, associated with the photosynthetic pigments, such as the light harvesting complex (LHCII) and the reaction centres of the PS II (Yamane et al., 1997). The results from the transient curves of the chlorophyll fluorescence, as well as the pigment analysis (Fig. 15) confirmed, that the degradation of these components from the photosynthetic apparatus started in NT even after 4°C. The change in the position of the exponential part of the curve before J to the left (quicker reaching of J) in both tolerant populations (T and Rila) is most probably related to slowing of the electron transport at the primary electron acceptor Q_A due to structural and functional changes in PS II (so-called K peak). The increase of the value of F_J in NT was observed in other studies, as well (Nellaepalli et al., 2012) and was thought to be related to the higher accumulation of reduced Q_A, most probably due to the partial blocking of the electron transport after the reduced plastoquinone (PQH_2). In the main case, this higher activity of PS II as compared with PS I is the basic reason for the accumulation of PQH_2. This occurs most often after stress when the electron transport was partially obstructed, or in the case when end acceptors of electrons were not sufficient amount, after inhibition of end stages of photosynthesis (Szarka et al., 2012). In addition, literature data shows, that this accumulation of PQH_2 could be a reason for

the phosphorylation of the LHCII from the protein-kinase STN7 in condition of intensive light (Zito et al., 1999). The phosphorylation of LHCII was observed after accumulation of reduced plastoquinone in *Arabidopsis*, as well, but without a relation to the light (Nellaepalli et al., 2012). Usually, the phosphorylation of LHCII leads to its dissociation from PS II and to its binding to PS I, so it is assumed, that this process protects plant from the excess light energy. After all, a migration of LHCII to the structures of PS I after chilling in *Arabidopsis* was not observed (Nellaepalli et al., 2012). The accumulation of PQH_2 facilitated the phosphorylation of LHCII after heat stress in *Arabidopsis*, as well (Nellaepalli et al., 2011). In our case with the applied cold stress at NT after 4°C and -7°C increase of the initial values of the fluorescence (F_O) was also observed, which was measured in other studies, for example in *Haberlea rhodopensis* after drought (Strasser et al., 2010) and *Arabidopsis thaliana* after cold stress (Mishra et al., 2011; Nellaepalli et al., 2012). The latter would probably be related to the separation of the antennae complexes from PS II (Yamane et al., 1995), due to accumulation of reduced forms of the plastoquinone (Toth et al., 2007), and also to physiological changes in the plant leaves (chloroplast orientation, change in turgor, decreased content of chlorophyll) (Strasser et al., 2010). In should be noted, that in plants from the NT population after 4°C the curve showed lower fluorescence in the points I and P (F_I, F_P), and the amplitude of the I-P phase was also decreased, as compared with the control temperature (22°C). Changes in I-P reflect the ability of the components, related to PS I for reduction, and these in F_I and J-I – the ability for reduction of the components from Q_B and PQ until PC (Bohme, 1978). Thus, changes in NT represent decreased reduction of the components after PS I, and the reasons for this could be the generation of ROS, as well as the activation of the cyclic electron transfer.

Our results showed very strong inhibition of the PS II after freezing in NT. This could be seen by the strong decrease of F_M after -7°C, reflecting the condition of the active reaction centres P680, whose amount was being sharply decreased. Except that, the electron transport after Q_A was almost completely disrupted, which was shown in the lack of the I-P phase, as well as from the intensities of the fluorescence in the points F_I, F_P, F_J, that had too close and not clearly differentiated values. This curve of fluorescence was much similar in appearance to the curve of plants, treated with diuron (DCMU), whose primary target is Q_A (Toth et al., 2005). In contrast to plant, treated with diuron, however, where the levels of the maximal fluorescence (F_M) were retained close to the control values, in the case of the cold stress in *A. alpina*, the values of F_M were not as much higher than the initial (F_O), which except the

blocking of the transport after Q_A showed a strong damage of PS II, as well. In plants from the tolerant population, the activity of PS II was not so significantly inhibited, like in NT, and in plants from Rila, this inhibition was even lower, as compared with T. The electron transport in the T population and the population from Rila was also preserved to a great extent, which could be seen from the retained phases J-I and I-P after 4°C and -7°C, but after -7°C the phase I-P was inhibited to a larger degree. In the plants from Rila, the phase I-P was strongly inhibited, but it was recovered after the recovery temperatures at 4°C and 22°C. In T the amplitudes of these phases were also recovered. After the stages of the recovery at 4°C and 22°C, respectively, plants from NT population were already desceased and this was shown by the transient curve as a fluorescence, with shape of a straight line and intensity, close to F_O. This residual fluorescence most probably is due to non-degraded chlorophyll molecules from the PS II or to the preserved, inactive centres of PS II. Although the activity of the photosynthesis and mostly PS II was decreased to half after treatment with -7°C in T, the normal appearance of the curve of functional photosynthetic apparatus was preserves in this population. The separate phases O-J, J-I and I-P were clearly distinguishable, although highly inhibited, as well as the intensities of the fluorescence F_J, F_I and F_P. After the stages of the recovery at 4°C and 22 °C, all phases of increase in the fluorescence, including the phase I-P in the plants from Rila were returned to their normal values, which is an indication for the recovery of the end acceptors from PS I and electron transport between the photosystems. On the other hand the intensity of the F_M in plants from both tolerant populations (T and Rila) was lower than the control, showing the slower recovery of the degraded antennae complexes and reaction centers of PS II, as well as the oxygen evolving complex.

- **Parameters of the prompt chlorophyll fluorescence**

Parameters of the prompt chlorophyll *a* fluorescence are present of Fig. 22 and 23, and were calculated on the basis of the transient curves from Fig. 20 and 21. The φ parameters (or the so-called quantum yields) represent the ratios of differences between the maximal fluorescence (F_M) from one hand, and the rest of the fluorescence stages on the other, as related to F_M. Thus, the separate quantum yields φ_{Po}, φ_{Eo}, φ_{Ro}, reflect the ratios $(F_M-F_O)/F_M = F_V/F_M$; $(F_M-F_J)/F_M$ and $(F_M-F_I)/F_M$, respectively. More precisely, they show the quantum yields from the primary photochemical reaction until the acceptor Q_A (φ_{Po}), the yield of the electron transport after Q_A (φ_{Eo}) and the reduction of the end stage electron acceptors from the acceptor side of PS I (φ_{Ro}).

Our results showed (Fig. 22), that with applying both types of cold stress, the values of parameters φ_{Eo} and φ_{Ro} were gradually decreased. These parameters showed low inhibition after 4°C, this inhibition being the most marked in φ_{Ro} in the NT population. After frost stress both parameters φ_{Eo} and φ_{Ro} were significantly decreased, much strongly in NT, as compared with T. Concerning φ_{Po}, this indicator retained within relatively stable borders (practically without any change) at 4°C, in T, as well as in NT. It should be noted, that after freezing φ_{Po} decreased to a much higher extend again in NT (30% inhibition in T and 75% in NT). It was shown out, that φ_{Ro}, reflecting the reduction of components from the side of PS I was more sensitive after both types of cold stress (Fig. 22). φ_{Ro} was sharply decreased at 4°C in both populations. After -7°C its values were decreased to about a half in T, and this decrease was much stronger in NT. Considering φ_{Eo}, even after 4°C higher sensitivity was registered in NT population (22% inhibition) and it was strongly increased after -7°C (73% inhibited), whereas the corresponding values in T remained relatively higher (only 3% inhibition after 4°C against 43% after -7°C). This tendency was the most strongly displayed in the parameter φ_{Ro}, in which the increase of its inhibition in NT after -7°C was 88% as compared with 61% in T. Thus, parameters φ_{Eo} and φ_{Ro} showed much higher sensitivity in NT after both types of stress. Other analysed parameters were the performance indexes (PI_{abs}, PI_{total}) (Fig. 22, 23), and the probability for electron transport beyond Q_A (ψ_o) and the thermal dissipation DI_O/RC, that was represented only for the Bulgarian population (Fig. 23). The performance indexes, themselves, reflect the efficiency for carrying the energy of captured electron from PS II until the intermediate electron transporters (PI_{abs}) and until the end electron acceptors from PS I (PI_{total}) (Strasser et al., 2010; Goltsev et al., 2010). Both parameters PI_{abs} and PI_{total} show the general condition of the photosynthetic apparatus and are considered to be especially sensitive regarding the changes in the photosynthetic apparatus after stress. PI_{total}, represented here for plants from both French populations was largely affected by the relative condition of plants because in its calculations parameters from both photosystems are included (Goltsev et al., 2014). PI_{total} is derived from φ_{Po} and ψ_o, the latter reflecting the probability for movement of electron beyond the primary acceptor Q_A, the relative amount of active reaction centres, as well as the parameter δ_o, that represents binding of electrons by the end electron transporters of PS I (Strasser et al., 2010).

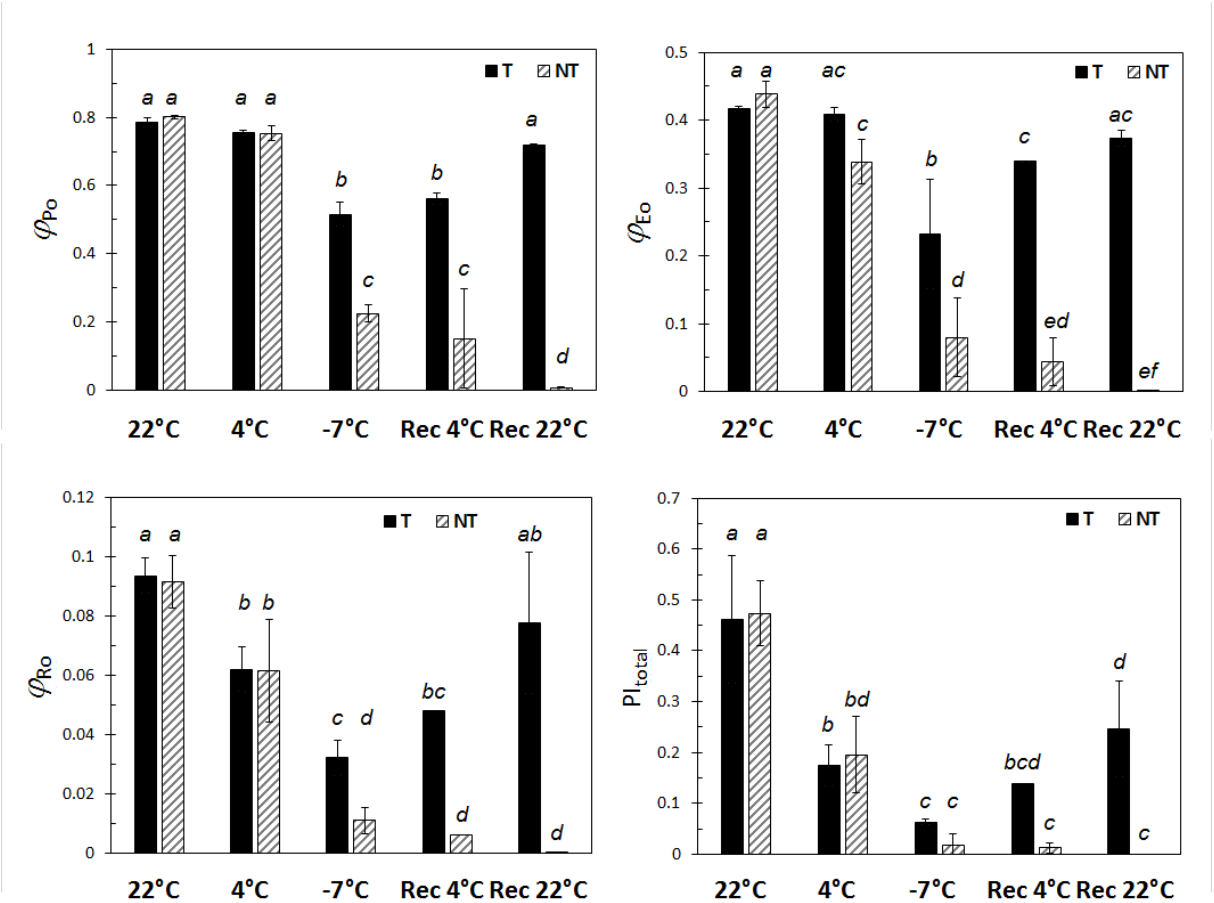


Fig. 22 Parameters of the prompt chlorophyll fluorescence (quantum yield, φ and the performance index, PI) of PS II. Measurement was carried out by the means of the M-PEA in the tolerant (T) and non-tolerant (NT) populations of Arabis alpina, in conditions of cold stress, chilling (4°C) and freezing stress (-7°C), as well as after a period of a subsequent recovery at 4°C and 22°C. Small letters display the statistically significant differences at P = 0.05.

The results from PI_{abs} were correlated very closely with the values from the net photosynthetic activity (uptake of CO_2), also shown in other studies (van Heerden et al., 2007). The results, presented on Fig. 22, illustrate the high sensitivity of the parameter PI_{total} in both populations T and NT. In contrast to the φ parameters of the quantum yield (especially φ_{Po}), the total performance index PI_{total} showed very strong inhibition even at +4°C in both populations, but this parameter was not able to recover after the minus stress in NT, as compared with T.

The parameters of the prompt chlorophyll fluorescence PF, obtained from the population from Rila mountain, showed values, close to those from the tolerant population T

from the Alps (Fig. 23). In the population from Rila mountain the strongest inhibition after frost (-7°C) was also observed in all parameters, except the dissipation of energy (DI_o/RC). Similarly to the plants from both French populations, in plants form Rila mountain the strongest inhibition was observed in the performance index PI, and, respectively the highest preservation of values and insensitivity to low temperatures, in the parameter φ_{Po}.

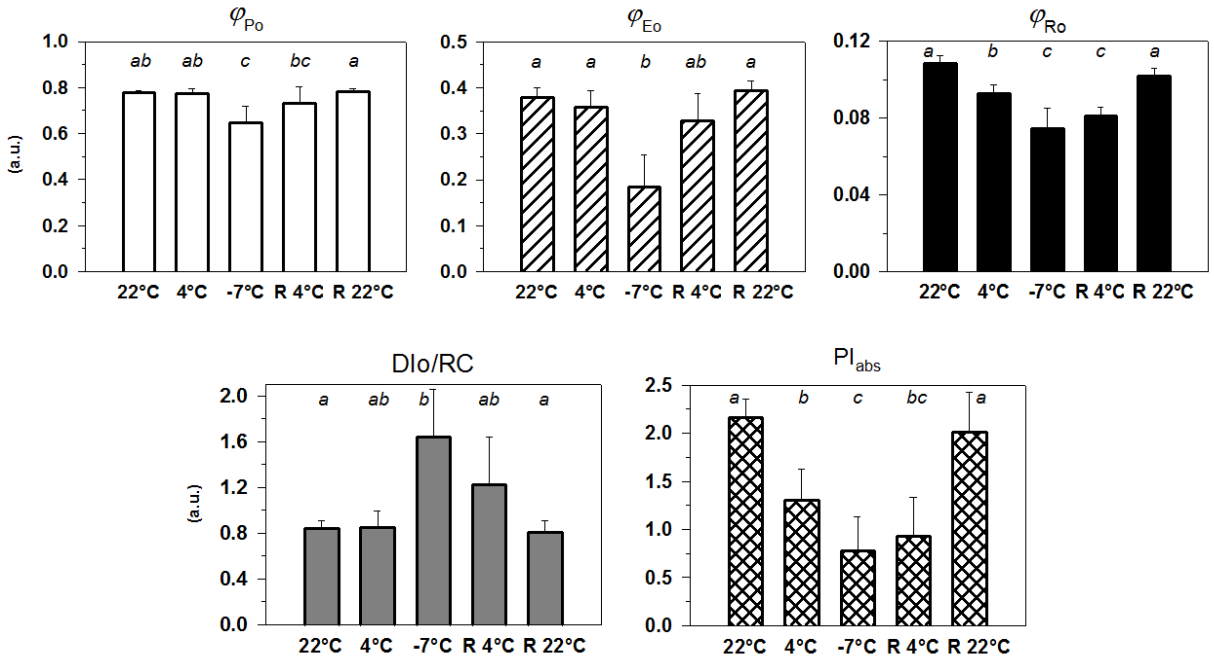


Fig. 23 Parameters of the prompt chlorophyll fluorescence (quantum yield, φ and performance index, PI) of PS II of intact plants of Arabis alpina, wild type from the area of the Seven Rila Lakes. The measurement was carried out by the means of the device M-PEA in conditions of cold stress, respectively chilling (4°C) and freezing stress (-7°C) and after a period of a subsequent recovery at 4°C and 22°C. Small letters show statistically significant differences at P = 0.05.

Results from this population showed, that parameters φ_{Eo} and φ_{Ro} (and especially PI_{total}), reflecting quantitative indicators of events, further away from PS II, were affected more strongly by the cold stress (Fig. 22).

Similarly to the French tolerant population T, in plants from Rila mountain the quantum yields were decreased to approximately similar levels after 4°C. This showed, that light phase of photosynthesis was not so strongly affected after this type of cold stress. The analysis of parameters of prompt chlorophyll fluorescence in the studied wild type plants from the area of the "Seven Rila Lakes" showed low levels of inhibition of the light phase of

photosynthesis (Fig. 23). These results were close to the results from the French population T (Fig. 22). From the graph could also be seen that after freezing the flux of electrons between both photosystems was more strongly inhibited in NT, even after 4°C. Results, obtained from the parameters of the induction curves in tolerant and non-tolerant populations of *A. alpina* after chilling and freezing showed, that components of the photosynthetic apparatus, closer to PS I, showed sensitivity to both types of cold stress, but the sensitivity was much stronger in the non-tolerant plants NT.

The parameters of the prompt chlorophyll fluorescence PF, obtained from the population from Rila mountain showed values, closer to those from the tolerant population T from the Alps. In the population from Rila the strongest inhibition of the parameters after frost (-7°C) was observed in every one of the parameters, except the energy dissipation (DI_o/RC). From the latter could be judged about the energy, which is not utilized, but dissipated form the PS II. Other analysed parameters, represented for the Bulgarian population were the total performance index (PI_{total}), as well as the probability for the electron transfer beyond Q_A (ψ_o). Like in the population from the Alps, in the population from Rila mountain the strongest inhibition was observed for the performance index PI_{abs}, and the insensitivity to low temperatures of the values of parameter F_V/F_M was mostly retained, respectively. From the obtained results could be concluded that plants from the Bulgarian population behaved similarly to the tolerant (T) to frost plants, isolated from the passway of Col du Galibier. In the other parameters of prompt chlorophyll fluorescence of PS II (φ_{Eo}, φ_{Ro}), this tendency of the results from the French populations was preserved, and namely the higher sensitivity of indicators, related to PS I. A higher sensitivity of the components from the electron transport of PS II located closer to PS I, is possible to follow from this.

- **Modulated 820 nm light reflection**

The modulated 820 nm light reflection (MR) is an indicator, reflecting the quantity of the reduced/oxidized forms of the plastocyanin (PC/PC^+), that is responsible for about of 30% from the reflection (Oja et al., 2003) and the reaction centre of PS I ($P700/P700^+$), which itself is responsible for about 70% of this reflection. It must be noted, that M-PEA has the possibility for simultaneously measurement of the PF, as well as the MR_{820}, and thus it could register changes in both PS II and PS I simultaneously. When a comparison of both parts of the MR-curve is made (Goltsev et al., 2010; Strasser et al., 2010), it could be noted, that the decreasing part of the curve (phase of the oxidation of PC and the reaction centre of PS I)

matches with the J-phase in PF (accumulation of reduced forms of Q_A). Thus in this stage both photosystems are being oxidized, the oxidation of PS II is registered by PF and the oxidation of PS I by MR_{820}. Analogically the increasing part of the curve of MR_{820} (the reduction of PS I) matches with the stage I-P in PF (the stage, where the donated electrons from PS II reach PS I). MR-curve in its upper part, corresponds to the inclusion of the newly synthesized compounds (ATP, NADPH) from the light phase in the cycle of Calvin-Benson (the phase of the carbon reactions) (Stirbert et al., 2013; Goltsev et al., 2014). On Fig. 24 and 25, MR-curves of plants from the populations from the French Alps and the mountain Vercors (Fig. 24, T and NT), as well as plant population from Rila mountain (Fig. 25, I and II) were represented, respectively. As a measurement for the oxidized/reduced forms of P700 and PC, curves of MR_{820} reflect the degree of the oxidation (decreasing part of the curve) and the reduction (increasing part of the curve) of PS I. The most intense oxidation (removal of electrons) from PS I was observed to the highest extent at 22ºC (corresponding to the maximal "depth" of the curve), which corresponds to the highest percentage of oxidized forms of $P700^+$ and PC^+ to their equivalents. With the increase of the cold stress a tendency towards a decrease in the level of oxidation of P700 was observed. Reaching the maximal values of the oxidation of P700 and PC (when highest amount of P700 and PC were present in oxidised state) their reduction was observed as well, which could be realized again only if the components are in a functionally active state.

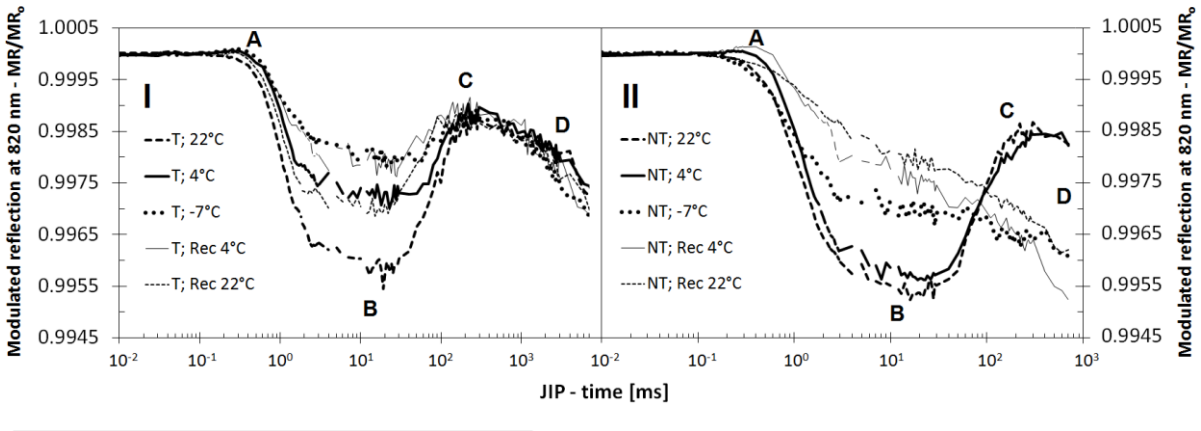


Fig. 24 Induction curves of the modulated reflection registered by the means of M-PEA at 820 nm as a measurement for the activity of the PS I. The study was carried out with individual plants from the tolerant (I) and the non-tolerant (II) populations of Arabis alpina in conditions of cold stress (chilling, 4ºC and freezing stress, -7ºC) and after a period of subsequent recovery at 4ºC and 22ºC.

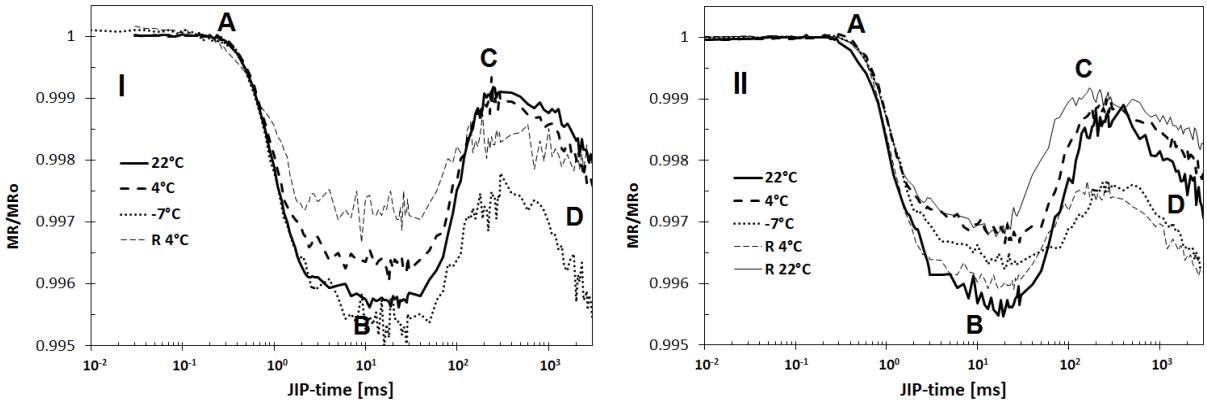


Fig. 25 Induction curves of the modulated 820 nm light reflection, as measured in A. alpina plants from Rila mountain: ***I)*** *leaves from intact wild plants, collected from Rila mountain.* ***II)*** *leaves from plants, grown from seeds in controlled laboratory conditions. Plants were treated with low temperatures (chilling, 4°C and freezing, -7 °C) and recovered at 4°C and 22°C, R - recovery.*

For example, the oxidation of tolerant plants could be clearly followed (in the fast part of the modulated reflection, the stage from A to B on the graphs) as well as their reduction afterwards (the slower part from B to C). The lowest point from the curve (point B), represents the reaching of equilibrium between oxidation and re-reduction of P700 and PC. Fig. 24 shows this point was located in its lowest position at the temperature of the control (22°C). The subsequent treatment with low temperatures (4°C, -7°C) led to the repositioning of this lowest point from the curves in upward direction. In *A. alpina* plants from T population the point B moved upwards to a greater extent than in NT after chilling at 4°C. The reaction of plants from this population to the decrease in the temperature, however, was represented as a decreased ability for reduction and was described by MR_{820} as a curve without this secondary increase. The treatment with -7°C (freezing stress) was a factor, clearly demonstrating the difference between both populations of *A. alpina* (Fig. 24). The measurement of the activity of PS I by the means of MR after treatment with -7°C showed clearly the resistance of T-population to this temperature, as compared with NT. In T a second reduction (re-reduction) in the phase B-C after freezing was observed, showing the retained activity of the PS I and the flux of electrons from PS II, whereas in NT this re-reduction in the phase B-C was missing. The preservation of the activity in PS I (although to a much lower degree, than at 22°C) could be seen in T as a secondary recovery of the increasing part of the curve (reduction of PC and the reaction centre of PS I), which was missing in NT. After frost stress, in the period of recovery (4 days at 4°C and 4 days at 22°C), the curves of MR in tolerant

plants almost returned to their appearance in the control plants (Fig. 24). In population NT after freezing a higher damage of PS I was observed, which was represented only in the decreasing part (the process of oxidation), but to a much lower degree as compared with 4°C and at 22°C. In contrast to T, it was not able to return to its normal appearance and increasing curve (phase of the reduction of PS I).

The MR_{820} curves of the plants from Rila mountain were presented on Fig. 25, panel I and II. After the cold stress they showed characteristics, similar to the T population concerning the response of PS I, as well as some differences. Their MR-curves showed tendencies, similar to T of increasing in the values of the minimal oxidation of P700 and PC in p. B after 4°C. This was represented as a lower degree of oxidation of P700 after the applied chilling stress as compared with the control (Fig. 25). In the literature data were showed also other stress effects (e.g. from drought in *Haberlea rhodopensis*, pea after heat stress), where the curve of MR_{820} was affected in a similar way (Strasser et al., 2010; Oukarroum et al., 2013). The oxidation of P700 in plants from Rila mountain, however, was inhibited to a lower degree, as compared with T (Fig. 24). Like in T, in plants from Rila the ability of PS I for re-reduction was shown after applying the freezing stress at -7°C. In contrast to plants from T population, where the oxidation of P700 was inhibited to a higher level (Fig. 24), in the plants from Rila even a low stimulation of oxidation was observed (Fig. 25), showing the accumulation of reduced forms of plastocyanin and plastoquinone in T after decreasing the temperature, whereas in plants from Rila the accumulation of these reduced forms occured only after 4°C. It should be noted, that accumulation of reduced forms of PQ and PC in conditions of stress was shown in the literature data for some plants (Nellaepalli et al., 2011; 2012). It is possible this accumulation to be due to the higher amounts of reduced NADPH, which often occurs in the conditions of decreased uptake of CO_2 (Szarka et al., 2012). Amplitudes of re-reduction in the plants from Rila (Fig. 25, phase B-C) were almost half lower than the control, in the same way, like in T. Amplitudes of oxidation in the phase A-B, however, were much closer to the control values (Fig. 25) and higher, than the values from T (Fig. 24, T), approaching the NT values (Fig. 24, NT). These processes were observed on Fig. 25 as lower values of the curve in the phase B, as well as two-times lower level of re-reduction (phase C), in contrast to T, where in the phase of re-reduction P700 and PC were re-reduced to their initial values (before p. A). It is well-known, that PS I is a large source of reactive oxygen species in stress conditions due to the lack of enough $NADP^+$ as a substrate (Apel and Hirt, 2004). It is possible that the higher level of oxidation (phase A-B) to be due to

such loss of electrons from PS I, causing the formation of ROS when the process for photoreduction of the oxygen from the FeS cluster of PS I has been activated (Schansker et al., 2005; Stirbert et al., 2013).

After -7^{o}C the retained activity of PS I (although to a much lower degree, as compared with 22^{o}C) could be seen in T as a subsequent recovery of the shape of the curve to upwards (reduction of PC and the reaction centre of PS I), which was absent in NT. This is due to the disruption of the electron transport between both photosystems. Experiments were carried out at room temperature (look materials and methods), and thus this blocking of the electron transport was due to long-lasting structural damages in the photosynthetic apparatus and was not a momentary change due to the low temperatures. As a result, PS II stopped donating electrons to PS I and, although PS I retained its ability for oxidation to some extent (but the speed of oxidation being decreased much stronger), it lost completely its ability for re-reduction. In tolerant plants after frost, in the period of recovery (4 days at 4^{o}C and 4 days at 22^{o}C), curves of MR almost returned to their appearance from before the stress. The phase B, however continued to have higher values, as compared with the control even after recovery at 22^{o}C. At the same time in NT the damages of PS I were increased, and this was represented as a lower speed of oxidation of PS I (the curve had a much more steep slope). In contrast to T, in NT the curves lost their phase of re-reduction (B-C), and the slope of the curve in the phase of oxidation became even steeper, corresponding to the destruction of more and more reaction centres of PS I (Fig. 25).

05.06. HPLC analysis of the content of phytohormones

- **Content of ABA and the compounds from the metabolism of ABA**

Being well-known from the literature, ABA is responsible for the induction of the state of physiological dormancy in plants, it is also related to the decreased vital activity after stress and it is the main hormone, together with ethylene, responsible for the transition of the perennial plants from the moderate climate during the winter season into a state of dormancy (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005).

In the current work the content of ABA and products of its catabolism (9-hydroxi-ABA, 9′OH-ABA, phaseic acid, PA; dihydrophaseic acid, DPA and the glucose ester of ABA, ABA-GE) were studied. Initially ABA is oxidised to PA. The transition state of this degradation is the 8′ –OH-ABA. In our work the content of the 9′-OH-ABA, was determined, which represents the transition state in the oxidation of the 9′-C atom of ABA to neophaseic

acid (neoPA) (Okamoto et al., 2011). (8′,9′) OH-ABA are intermediate compounds in the oxidation of ABA to PA (and neoPA, respectively) and are relatively unstable, thus oxidizing spontaneously to the next products of the reaction. The dihydrophaseic acid (DPA) is the end oxidation /degradation/ product of ABA. In contrast to PA, that has the specific for ABA activity /closing of stomata/, DPA does not have any ABA activity (Walton and Li, 1995).

Our results showed, that the ABA content after treatment with cold stress (chilling stress 4^{o}C) was increased (almost two-fold) in plants from the tolerant population, as well as in plants from the non-tolerant population of *Arabis alpina* (Fig. 26). After the treatment with minus temperatures the content of ABA was decreased from the values at 4^{o}C in both populations T and NT, retaining its values, close to control at 22^{o}C. Similar behaviour was observed for the direct products of degradation (oxidation) of ABA: 9′OH-ABA and PA (Fig. 26), the content of PA was increased mostly after 4^{o}C and it remained almost unchanged after -7^{o}C in both populations. The content of 9′OH-ABA was comparatively lower in both populations studied, and was also increased after cold stress (4^{o}C). It is clearly seen from the results, that the content of PA as first product from the degradation of ABA was higher, than the ABA itself. These tendencies were observed in the plants from T population of *Arabis alpina,* as well as in the NT population. As regards DPA, representing the final degradation product from the metabolism of ABA, significant difference in both populations was observed. In the *A. alpina* plants from the tolerant population T, the content of DPA was the highest of all studied products from the catabolism of ABA, or 2.269 nmol/g DW in the control temperature at 22^{o}C and did not change substantially after treatment with 4^{o}C and -7^{o}C. In plants from the NT population only traces of DPA were observed, its content being significantly lower as compared with the content of DPA in plants from the tolerant population.

The content of ABA is indicative for the degrees of stress, to which the plant was exposed. After stress its content usually increases, depending on the strength of the stress factor, for example after drought in *Zea mays* (Beardsell and Cohen 1975) showed close relationship between the water potential of the leaf, the stomatal conductance and the content of ABA. The higher concentration of the products from degradation of ABA indicated a stronger metabolite activity of this compound in the plant organism. This difference between the metabolism of T and NT could be best seen in the final product of ABA degradation, DPA, which was the compound from the metabolism of ABA, represented in the highest amount in T, whereas in NT it was almost missing. It is hard to answer what could be the

reason for this, but most probably, it was due to the higher degree of synthesis, metabolism and degradation of ABA in T.

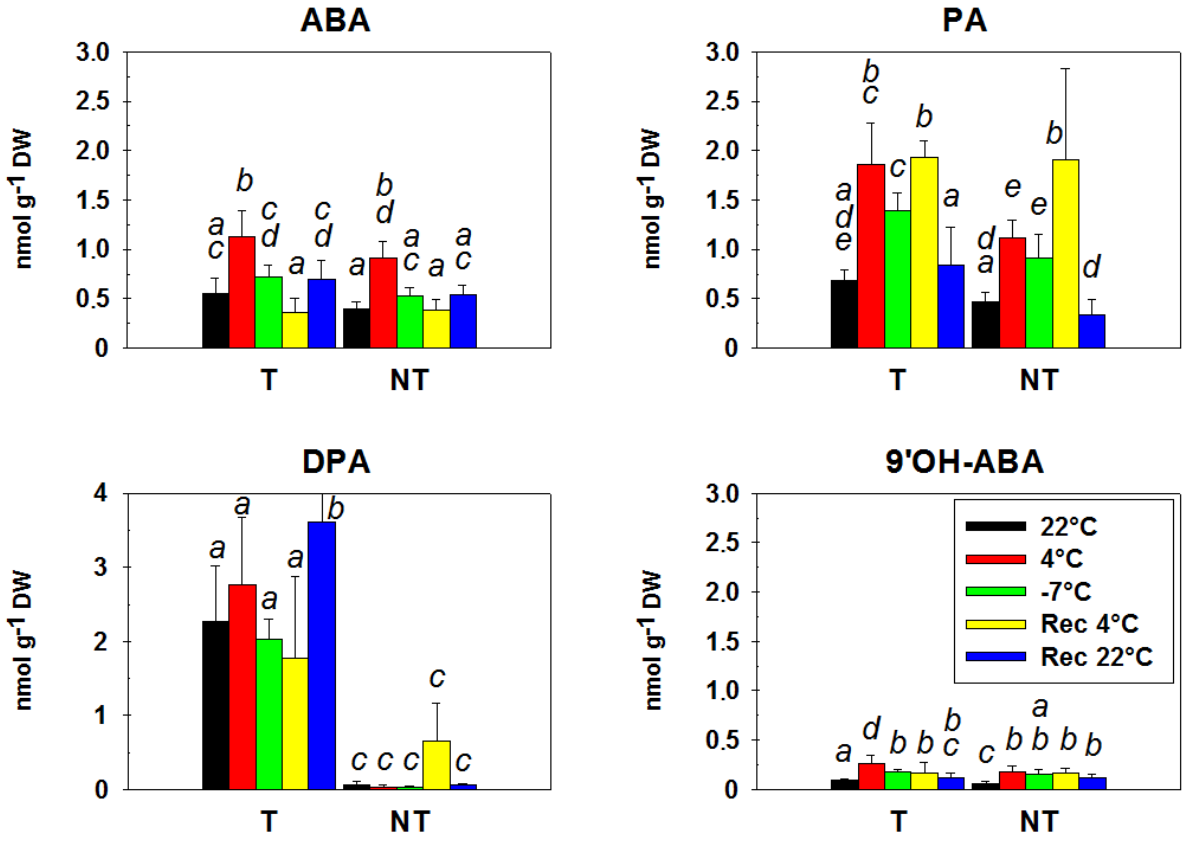


Fig. 26 Content of abscisic acid (ABA) and its degradation /oxidized/ forms in tolerant (T) as well as non-tolerant (NT) populations of Arabis alpina, in conditions of cold stress (chilling +4°C and freezing stress -7°C): A) ABA; B) phaseic acid (PA); C) dihydrophaseic acid (DPA); D) 9' ABA hydroxide (9'OH-ABA). Small letters indicate the statistically significant differences at P = 0.05.

- **Total content of the main groups of cytokinins (CK)**

Like it was mentioned earlier in this dissertation work, CKs have diverse functions in the plant organism, the most important of which being the stimulation of cell division and the growth of the meristem shoots. The ratio between cytokinins and auxins determines the growth of the root and the shoot parts of the plant (Skoog and Miller, 1965). CKs are hormones, stimulating multiple basic physiological processes in plants, in contrast to ABA,

being a hormone of the stress and the physiological dormancy. In addition, CK regulate the photosynthetic activity and they exert effects upon the circadian rhythm of plants, as well.

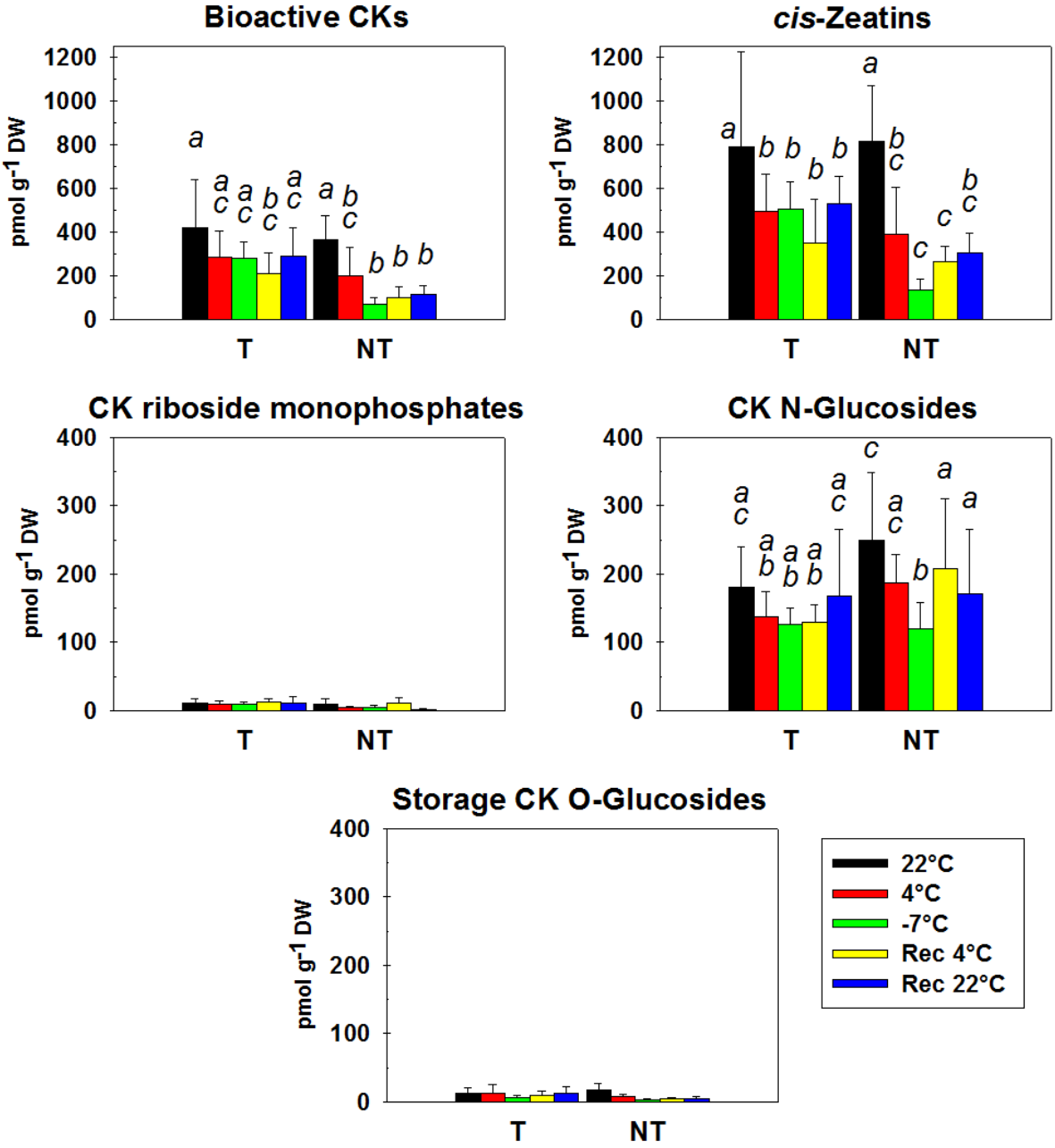


Fig. 27 Content of: A) bioactive cytokinins; B) cis-zeatins; C) CK riboside-monophosphates; D) CK N-glucosides; E) CK O-glucosides in tolerant (T) and non-tolerant (NT) populations of Arabis alpina, in conditions of cold stress (chilling +4°C and freezing stress -7°C). Small letters indicate statistically significant differences at P = 0.05.

By applying the modern analytical methods of HPLC/MS-analysis, the content of the five main groups of cytokinins was studied. The first included the biologically active CK *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), isopentenyladenine (iP) and isopentenyl riboside

(iPR), dihydrozeatin (DHZ) and dihydrozeatin riboside (DHZR). The second group consisted of the considered inactive *cis*-zeatins, including *cis*-zeatin (cZ), *cis*-zeatin riboside (cZR), *cis*-zeatin monophosphate (cZRM) and *cis*-zeatin-O-glucoside (*cis*-ZOG). The rest three groups consisted of the CK riboside monophosphates that are the first products from the biosynthesis of CK, the storage forms CK-O-glucosides and the inactive CK-N-glucosides. The main groups of cytokinins were represented as a sum, graphically, according to their biological role, as well as separately in groups of cytokinins from each of these groups.

The content of the described in the upper section five main groups of CK, depending on the temperature of development or treatment of plants was presented on Fig. 27. Content of the biologically active CKs at control temperature (22^{o}C) was close in both populations of *Arabis alpina* and was 0.422 nmol/g DW and 0.364 nmol/g DW in populations T and NT, respectively (Fig. 27, A). In tolerant population T, concentration of biologically active CKs remained almost unchanged after the low temperature chilling stress at 4^{o}C, as well as after the stress at -7^{o}C with values of 0.286 nmol/g DW and 0.279 nmol/g DW, respectively. In the non-tolerant NT population the content of biologically active CK was progressively decreasing after 4^{o}C to 0.202 nmol/g DW and four times (72 pmol/g DW) after the treatment with -7^{o}C. During the period of the subsequent recovery at 4^{o}C and at 22^{o}C the cytokinin content was returned to the concentrations after the treatment with 4^{o}C, but it could not recover to the amounts of the control temperature of 22^{o}C from before the stress.

The behaviour of the *cis*-zeatins was very similar to that of the biologically active cytokinins and was presented on the next graph from the figure (Fig. 27, B). It could be well seen, that the changes in the content of the *cis*-zeatins repeated the tendency of changes in the concentration of the biologically active cytokinins after applying the cold stress. It should be noted, that the content of *cis*-zeatins was about two times higher than that of the biologically active cytokinins at the control temperature of 22^{o}C. In control plants there was almost no difference in the content of the *cis*-zeatins between populations T and NT (0.791 nmol/g DW and 0.812 nmol/g DW respectively in both populations). After 4^{o}C the content of *cis*-zeatins was decreasing to 0.495 nmol/g DW in T and to 0.388 nmol/g DW in NT. After applying of -7^{o}C the concentration remained almost the same in T (0.506 nmol/g DW), but was decreased to 0.145 nmol/g DW in NT. After the subsequent treatments at 4^{o}C and 22^{o}C the content of *cis*-zeatins was recovered to the levels from the first treatment with 4^{o}C.

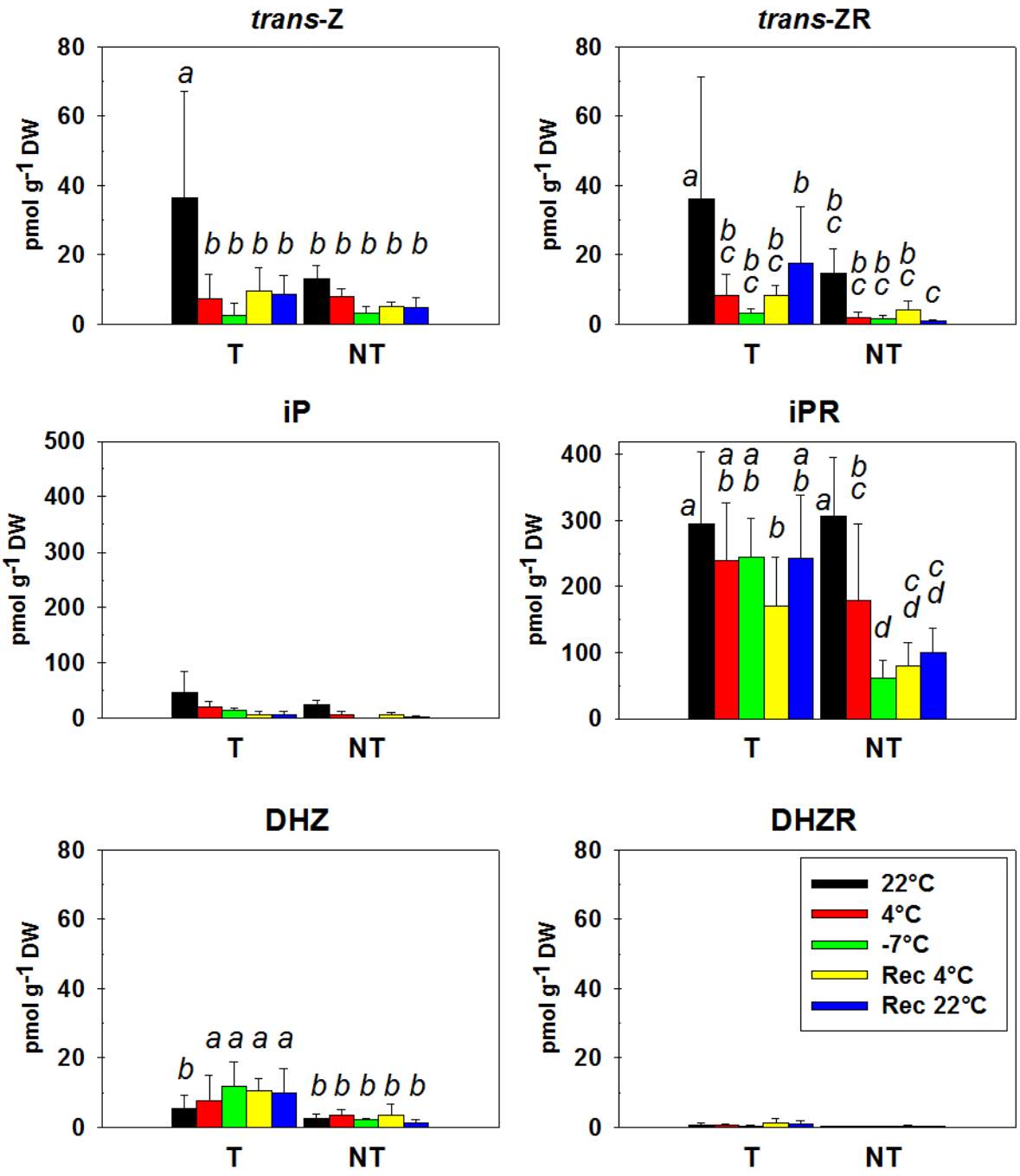


Fig. 28 *The content of the biologically active cytokinins: A) trans-zeatin (trans-Z); B) trans-zeatin riboside (trans-Z R); C) isopentenyladenine (iP); D) isopentenyladenine riboside (iPR); E) dihydrozeatin (DHZ); F) dihydrozeatin riboside (DHZR), respectively in the tolerant (T) and non-tolerant (NT) populations of Arabis alpina, in conditions of cold stress (chilling +4°C and freezing stress -7°C). Small letters indicate statistically significant differences at P = 0.05.*

The content of the other two groups of CKs, CK riboside monophosphates, that include the isopentenyladenine riboside monophosphate and *trans*-zeatin riboside monophosphate, as well as the total amount of the storage O-glucoside forms of CKs was negligible small as compared with the levels of the rest cytokinins (not more than 20 pmol/g DW) (Fig. 27 C, E). Thus, we will not describe them.

The content of N-glucoside cytokinins was presented on Fig. 27, D. More generally speaking, it could be said, that it was close to the content of the biologically active CK and followed the same tendency of changes, but only in T population. In the control temperature of 22ºC their content was 0.180 nmol/g DW in T and 0.250 nmol/g DW in NT. At 4ºC their level in T was decreasing respectively to 0.138 nmol/g DW and 0.187 nmol/g DW in NT. After -7ºC their content was decreased more strongly in NT as compared with T (0.127 nmol/g DW and 0.119 nmol/g DW, respectively in both populations). It could be seen, that after treatment with negative temperature levels in T were retained, whereas in NT they were decreased around two times. After the subsequent recoveries at 4ºC the content in T as well as NT returned in the values from the first treatment with 4ºC, and in the recovery at 22ºC in T values were recovered in concentrations, close to the control, whereas in NT the content during the recovery at 22ºC remained close to the concentrations at 4ºC.

- **Content of individual types of cytokinins from the different groups**

Content of the biologically active cytokinins

It could be clearly seen, that the content of biologically active cytokinins *trans*-Z and *trans*-ZR (Fig. 28) was quite low (below 40 pmol/g DW), the content of both types was decreasing in a similar way after treatment with low temperatures. The concentrations of *trans*-Z and *trans*-ZR in control plants (at 22ºC) in the T-population were practically the same (about 37 pmol/g DW). In population NT the content of these cytokinins were also very close (around 15 pmol/g DW), but two times lower than T. After the period of chilling (4ºC), the concentrations of *trans*-Z and *trans*-ZR were sharply decreased to 7.7 pmol/g DW (*trans*-Z) and 8.4 pmol/g DW (*trans*-ZR) in T population. In NT (after 4ºC) the content of *trans*-Z was 8 pmol/g DW, and *trans*-ZR was 2 pmol/g DW. The content of both types biologically active cytokinins was decreased to an even greater extent after -7ºC reaching 2.9 pmol/g DW for *trans*-Z in T and 3.4 pmol/g DW in NT. At the same time, the concentration of *trans*-ZR was decreasing to 3.4 pmol/g DW in T and 1.7 pmol/g DW in NT. After the first period of

recovery at 4°C the concentrations of *trans*-Z and *trans*-ZR reached the values from before the treatment with -7°C. After the recovery at 22°C, the concentrations were not able to reach the initial control values. Concerning the biologically active CK from the isopentenyladenine group (iP and iPR) (Fig. 28), a substantial difference in their content was observed. iPR was reaching 300 pmol/g DW (making it the bioactive cytokinin with the highest content from the current study in *Arabis alpina)*, whereas the content of iP was insignificantly low (15-20 pmol/g DW). However, on the background of these low concentrations, a decrease of iP in the NT population was observed. It should be noted, that changes in the content of iPR were similar to those of the *cis*-zeatin type CKs (Fig. 27). The concentration of iPR at the temperature of the control (22°C) was very close in both populations (296 pmol/g DW in T and 308 pmol/g DW in NT). After cold stress in 4°C, the content of iPR was decreasing in both populations, but to a much higher degree in NT (241 pmol/g DW in T and 181 pmol/g DW in NT). During treatment with negative temperature -7°C this difference was increasing even more, because the concentration in T remained the same (247 pmol/g DW), and in NT it was decreased drastically to 63 pmol/g DW. After the period of recovery, the content in T was retained to the values form 4°C, and in NT it was increasing, without reaching the content from before the treatment with -7°C. The concentrations of DHZ and DHZR were the lowest from all bioactive cytokinins, or below 20 pmol/g DW (Fig. 28). This content remained relatively constant after treatment with low temperatures with a tendency of increasing, whereas in NT, it was comparatively low, as well as for the DHZR (Fig. 28).

The content of the total amount of *cis*-zeatins (Fig. 27) and, more specifically *cis*-ZR (Fig. 29) was the highest of all studied CKs (almost two times higher than the biologically active CKs at temperature of 22°C). The content of *cis*-ZR itself was also from two to three times higher than *cis*-Z. In contrast to NT, *cis*-ZR was not changing under the effect of both low temperatures only in population T. In this sense, changes in *cis*-ZR under the effect of different temperatures were similar with those in iPR (see Fig. 28). Regarding the content of *cis*-ZRMP (the first product from the biosynthesis of *cis*-Z) and *cis*-ZROG (with storage functions) (Fig. 29), these forms of cis-zeatins were mostly decreasing after both types of cold stress in both studied populations of *A. alpina*.

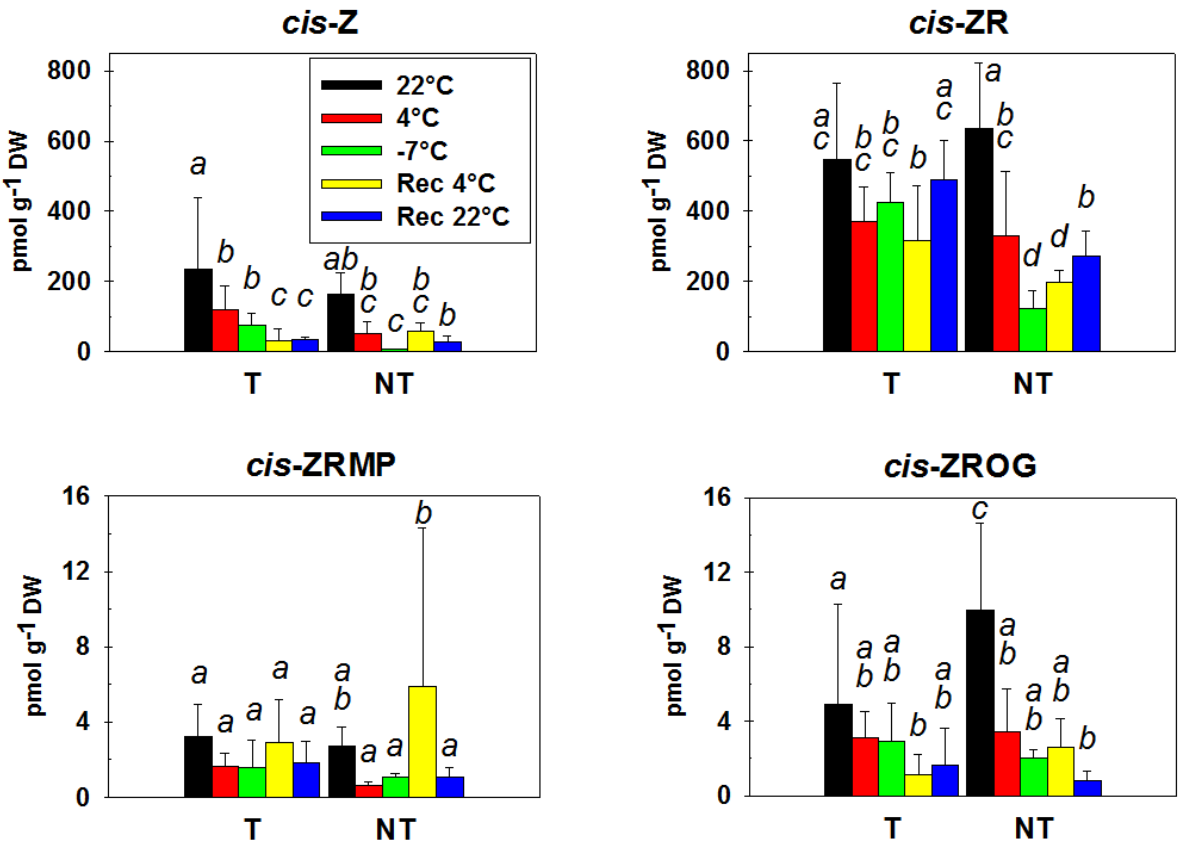


Fig. 29 Content of CKs from the cis-zeatin type: A) cis-Z; B) cis-ZR; C) cis- ZRMP; D) cis-ZROG in plants from the tolerant (T) and non-tolerant (NT) populations of Arabis alpina, after cold stress (chilling, +4°C and freezing stress, -7°C). Small letters indicate statistically significant differences at P = 0.05.

More generally speaking, in this current work the content of main cytokinins, the bioactive *trans*-zeatin, *trans*-zeatin riboside, isopentenyladenine and isopentenyl riboside, dihydrozeatin and dihydrozeatin riboside was studied. Concentrations of inactive *cis*-zeatins (including *cis*-zeatin, *cis*-zeatin riboside, *cis*-zeatin monophosphate, *cis*-zeatin-O-glucoside) were studied, as well as the storage forms O-glucosides and the inactive N-glucosides. *Cis*-zeatins were represented in the greatest amount, despite the widely accepted opinion, that they have lower activity than *trans*-Z and also the iP isoformes (Spiess, 1975; Schmitz and Skoog, 1972). In some species and systematic groups however, for example *Zea mays* (Veach et al., 2003) and *Oryza sativa* (Murofushi et al., 1983) their content is higher and was found that in these plants, in contrast to *Arabidopsis* the *cis*-CKs are almost with the same bioactivity as compared with *trans*-CKs. Changes in the content of *cis*-zeatins in T and NT populations of *A. alpina* after cold stress strongly resembled the changes in the biologically active CKs. In T their content was slowly decreased after 4°C, and after -7°C did not change. The content of

trans-zeatin and its precursor the *trans*-zeatin riboside was low and had values, very close to both cytokinins, similarly as in T, their content in the control was higher than NT (Fig. 28). After stress the content of both cytokinins was decreased in both populations, to a greater extent in NT. The isopentenyladenine was represented with insignificant values (Fig. 28). Its precursor, the isopentenyladenine riboside was the most strongly accumulated from all bioactive cytokinins and determined the behaviour of the total amount of the biologically active cytokinins. Its amounts in both controls (T and NT) were close, and after 4^{o}C they decreased very little to a similar level. After freezing however, the content in T was retained, whereas in NT it was decreased significantly (Fig. 28). Dihydrozeatin and dihydrozeatin riboside were represented with insignificant values. From the *cis*-zeatins only the *cis*-zeatin riboside was represented with significant amount, and its behaviour resembled the isopentenyladenine riboside, but the content was much higher (Fig. 29).

On the basis of the results presented here, it could be assumed, that cytokinins are involved in the formation of the tolerance to low temperatures (chilling and freezing) in *Arabis alpina.* It also could be possible that *cis*-zeatins have much greater significance as a whole in the life cycle of *A. alpina*, as compared with other plants from the Brassicaceae family, for example *Arabidopsis*. It should be interesting to note, that the *cis*-zeatins take place in the earliest development stages in *Arabidopsis*, where the ratio *cis*-/*trans*- zeatins changes drastically during a week after germination in the favour of *trans*-zeatins (Gajdosova et al., 2011). *Cis*-zeatins also have important role in the life cycle of grain crops (Poaceae), where they are accumulated after fertilization (Sykorova et al., 2008). Isopentenyladenin riboside and the *cis*-zeatin riboside were the cytokinins with the highest amount in *A. alpina* and it is logically to assume they are the biologically active forms in this plant. In the tolerant to -7^{o}C plants from population T, the content of these cytokinins did not change, in contrast to the non-tolerant, demonstrating their significance for the reaction of the plant to this type of stress. The amounts of the different cytokinins, as well as the ratio *cis*-/*trans*-zeatins is independent from the systematic groups and is not determined by some special charateristics of plants. Different ratio between *cis*-/*trans*-cytokinins could be found in lower or higher plants such as monocots (Parker et al., 1998; Veach et al., 2003), dicots (Nicander et al., 1995; Emery et al., 1998, 2000; Dobrev et al., 2002; Ananieva et al., 2004; Gaudinova et al., 2005; Malkawi et al., 2007; Stirk et al., 2008; Van Staden et al., 2010), as well as algae (Stirk et al., 2003; Ordog et al., 2004) and mosses (Von Schwartzenberg et al., 2007). In some mosses, for example, *cis*-zeatins are in a much higher content, but this is so in grain crops /Poaceae/ such

as rice and maize (Gajdosova et al., 2011). On the other hand between the monocots and dicots there exist species, having high, as well as low ratio of these phytohormones. It is possible such division in the cytokinin groups to have some evolutionary significance in the frames of the species themselves, considering this difference in the represented in the plant cytokinins can be observed between closely related species. For example in the Brassicaceae family as a whole *trans*-zeatins are represented with a significantly higher amount (Simpson, 2006, Gajdosova et al., 2011), but in *Arabis alpina cis*-zeatins were the most (about 65% of bioactive cytokinins), followed by the isopentenyladenines (30%), whereas the *trans*-zeatins were comparatively lower amount (not more than 5%), and the dihydrozeatins were insignificantly low.

- **Analysis of the endogenous content of phytohormones jasmonic acid and jasmonate-isoleucine**

The jasmonic acid (JA) and its derivatives methyl-jasmonate (MeJA) and jasmonat-isoleucine (JA-ile) are known as active mediators of the responses of plant cell to the multiple factors of biotic and abiotic stress, including wounding, herbivore insects, pathogens, drought and other types of stress (Ding et al., 2001; Wasternack, 2007; Kosova et al., 2012). Endogenous jasmonate content was measured by the means of HPLC/MS analysis.

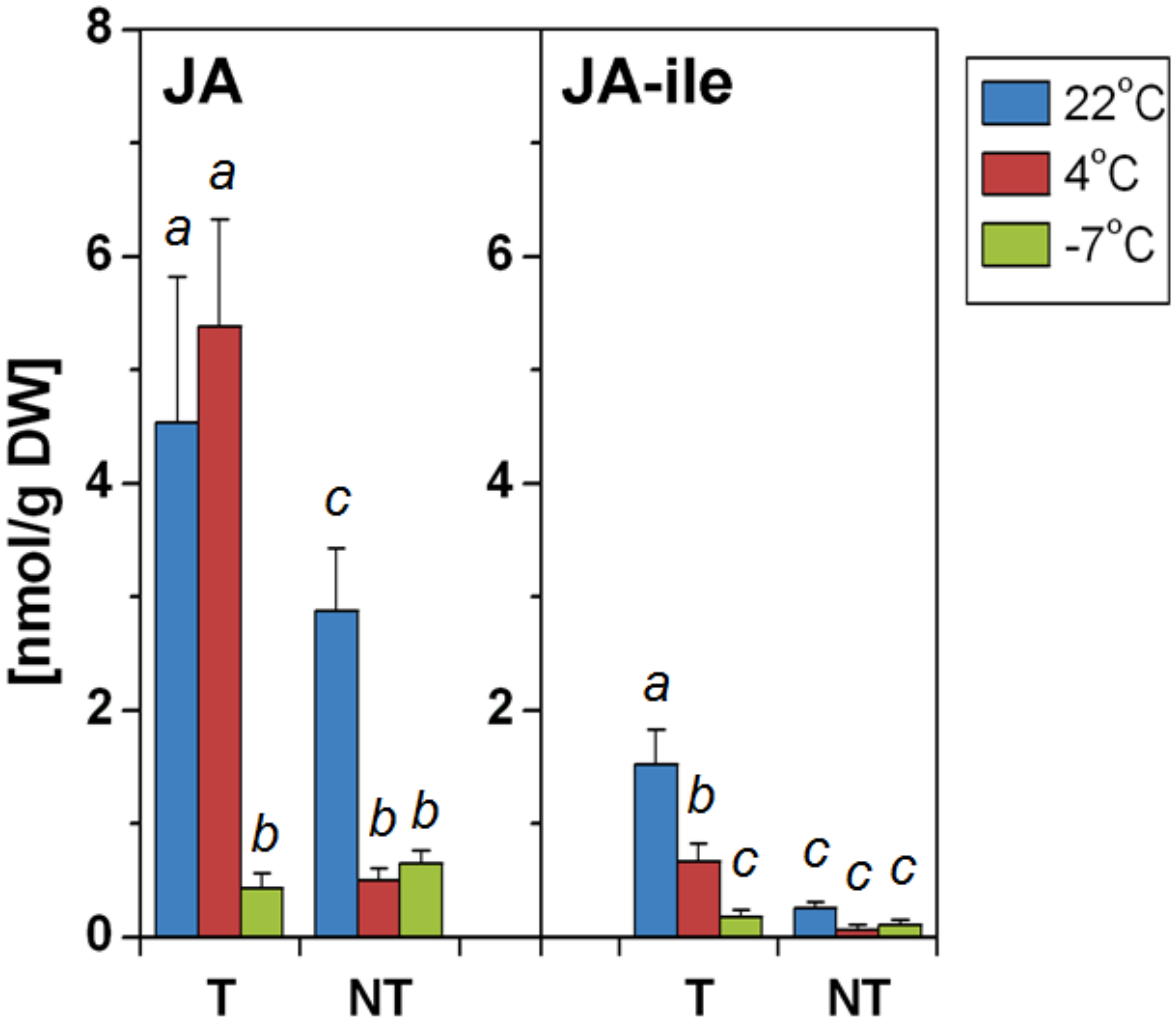


Fig. 30 Content of A) jasmonic acid (JA) and B) jasmonate-isoleucine (JA-ile) in tolerant (T) and non-tolerant (NT) populations of Arabis alpina to negative temperatures (-7ºC). Plants were grown at 22ºC (control conditions) and subsequently exposed to chilling (4ºC) and freezing stress (-7ºC) (see materials and methods). Small letters indicate statistically significant differences at P = 0.05.

Our results (Fig. 30) showed high content of JA (3-5 nmol g^{-1} DW), which was close to the amount of ABA (Fig. 26) in both studied populations. After chilling, the content of JA in plants from T population remained unchanged, in contrast to the 10-fold decrease of JA in NT-population (0,5 nmol g^{-1} DW). The content of the biologically active jasmonate-isoleucine (JA-ile) was significantly higher in the control temperature in T (1,5 nmol g^{-1} DW), as compared with NT (0,26 g^{-1} DW). JA-ile was significantly decreased after chilling in both populations, remaining relatively high in T population (0,67 nmol g^{-1} DW) as compared with NT (0.07 nmol g-1 DW). Treatment of plants with negative temperatures led to a very sharp decrease of JA and JA-ile in the both studied populations of *A. alpina*. The obtained results

showed, that JA and its amino acid conjugate JA-ile could be involved in the acclimation of *Arabis alpina* to low temperatures.

The content of jasmonic acid in T was higher compared to NT in control temperature 22^{o}C. At the same temperature the jasmonate-isoleucine was represented in significantly higher amount in T, as compared with NT, but lower than the jasmonic acid. The content of jasmonic acid was sharply decreased even after chilling in NT, whereas in T it was retained. The jasmonate-isoleucine was decreased about two-fold after chilling in T. After -7^{o}C in both populations the content of both oxylipins were significantly decreased. Oxylipin phytohormones, such as the jasmonic acid and the jasmonate-isoleucine are mainly related to the protection against biotic stresses (insects, pathogens and wounding). Some studies showed a relationship between these hormones and the tolerance to low temperatures. In wheat, the content of jasmonic acid and salicylic acid was increased after cold stress (Kosova et al., 2012). Furthermore, the concentration of the jasmonic acid was increased significantly in population of *Pinus pinaster* after applying of low temperature and drought (Pedranzani et al, 2007). The expression of the main enzyme from the biosynthesis of jasmonates LOX in kiwi (*Actinidia delicosa*) and *Caragana jubata* was regulated positively after treatment with low temperatures (Zhang et al., 2006; Bharwaj et al., 2011). Bharwaj et al. showed that LOX was activated from other factors as well, such as ABA, salycilic acid and even methyl-jasmonic acid, and therefore jasmonates could act by the amplification of the signal and actively participate in the regulation of the content of other phytohormones. Thus it could be highly possible for them to play a key role during stress, together with ABA. The mutual regulation between phytohormones is carried out by the means of the mechanism of cross-talk signalisation. The jasmonate and salycilate pathways, realising the plant response against insects and pathogenes, respectively, could be pointed out as a good example for mutuality in the biosignal pathways. These two plant regulators are antagonists and when genes of the one of them are expressed, genes for the transcription of the second are inhibited. The interaction by the means of cross-talk exists between jasmonates and other phytohormones, as well. Methyl-jasmonic acid affects negatively also the content of cytokinins in *Cucurbita pepo* (Ananieva et al., 2004). In addition, it is known that the oxylipin compounds form after lipid peroxidation (Berger et al., 2001, Demmig-Adams et al., 2012). In the presence of stress, biotic as well as abiotic or as a response to certain processes from the development of the plant (Bell et al., 1995) fatty acids from chloroplast membranes are being oxidised and after that oxylipins are being synthesized in peroxisomes (Chrispeels et al., 1999). In case of

insufficient antioxidant activity this process increases, due to accumulated ROS that additionally oxidise membrane lipids and thus oxylipin compounds form. These oxylipin compounds are the main precursors of jasmonates. Jasmonic acid, accumulating after stress acts inhibiting upon the process of photosynthesis, induces the degradation of chlorophyll and activates the synthesis of tocopherol, anthocyans and glutathione, that are extremely strong antioxidants (Szarka et al., 2012). Thus the content of jasmonates is regulated on the principle of the feed-back mechanism.

05.07. Transcriptional (qRT-PCR) analysis of genes from the metabolism of the phytohormones ABA and CK

- **qRT-PCR analysis of genes from the ABA metabolism**

Six genes from the metabolism of ABA were studied: 4 from the biosynthesis of ABA, two of which are isoforms from the gene family of *NCED* (9-cis-epoxycarotenoid dioxygenase, catalysing the formation of xanthoxin from violaxanthin), the two isoforms *NCED3* and *NCED5*, respectively. The other genes from the metabolism of ABA were *ABA1* (coding the enzyme ZEP – zeaxanthin epoxidase, catalysing the transformation of zeaxanthin into violaxanthin) and the gene *AAO3*, coding for the enzyme ABA-aldehydeoxydase, responsible for the last step from the formation of ABA. In addition, the gene *CYP707A1* responsible for the first step from the degradation of ABA, representing the oxidation of ABA to 8′ and 9′OH-ABA, as well as the gene *ABCG40*, coding for transporter of ABA from the apoplast to the cell, were studied. The data from the qRT-PCR analysis was represented on Fig. 31. It could be seen, that in almost all studied genes the rate of transcription increased after lowering the temperature, the strongest being the stimulation of the genes *NCED3* in both populations. The decreasing in levels of transcription of the genes *ABA1* and *AAO3* after treatment with -7^{o}C must be noted. The content of *ABA1* in the start of the experiment (22^{o}C) was low in both populations, and lower in NT. After 4^{o}C it was increased almost two-fold in T, as well as in NT. After frost the expression remained constant in NT, whereas in T it decreased to little above the level of the control temperature. The transcription of *NCED3* was lower than *ABA1* in both populations at 22^{o}C. In chilling conditions (4^{o}C) retained in T and was increased more than two times in NT. *NCED3* expression was significantly increased and was the highest after treatment with negative temperatures in both populations. The next studied gene was *NCED5*. Its expression at 22^{o}C (control conditions) was about two times higher than *NCED3* in both populations. Expression of *NCED5* was gradually increasing with

the decrease of the temperature. Regarding *CYP707A1*, which codes for an enzyme from the oxidation (degradation) of ABA, the stimulation of transcription was observed in both low temperatures. The data for the effect of low temperatures on the transcription of the gene *ABCG40* was of specific interest. With decreasing of the temperature, very strong increase of the transcription levels was observed in this gene only in T population. In control conditions (22ºC) levels of expression of this gene were significantly low and close to the levels of *CYP707A1*. After subsequent treatment with 4ºC and -7ºC the transcription of *ABCG40* in T population was increasing several-fold (10-20 times), in contrast to the NT population, where it did not change. These data show the participation of this transport protein for ABA preferably for the T population, as a possibility for the acquisition of tolerance to cold stress in this population.

The analysis of expression of genes from the ABA metabolism showed, that after cold stress the expression of most genes increased in both populations. The expression of these genes increased similarly to the described by Baron et al., 2012. The increase in the content of mRNA from the gene, coding for *NCED3* was the highest. This is the enzyme that synthesizes the first compound with ABA activity, xanthoxin, as well as the first compound from the ABA metabolism, synthesized outside chloroplasts. In contrast to *Arabis alpina*, in *Arabidopsis* the expression of this gene was decreased in leaves after treatment with 0ºC (Baron et al., 2012). The other isoform of this gene, *NCED5*, was increased in *Arabidopsis* as well as *Arabis alpina* after treatment with low temperatures. The expression of gene for the first step of degradation of ABA, *CYP707A1*, was also increased substantially even after chilling (4ºC) in both populations. Results correlated with the content of ABA and the degradation products of ABA, which increased after cold stress.

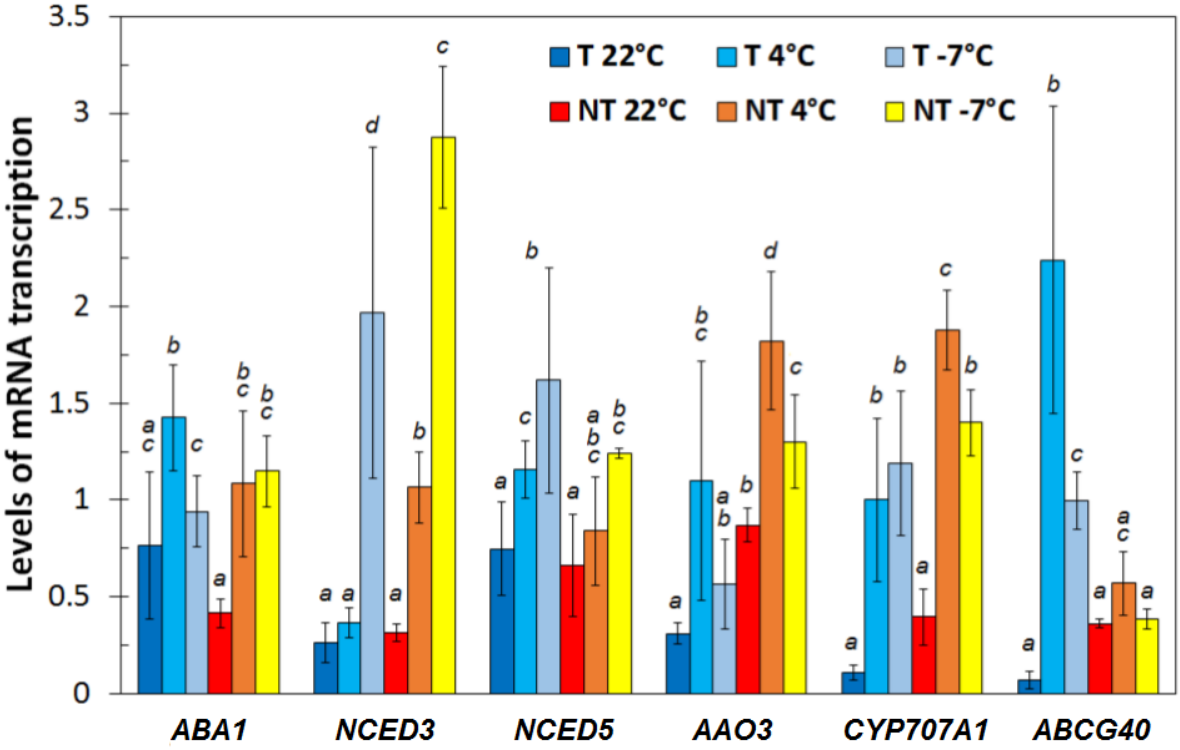


Fig. 31 qRT-PCR analysis of genes from the biosynthesis (ABA1, NCED3, NCED5, AAO3), degradation (CYP707A1) and the transport (ABCG40) of ABA. Total RNA sample, isolated from individual plants of the tolerant (T, GAL-T) and non-tolerant (NT, F005-25) populations of Arabis alpina, in conditions of cold stress (chilling +4°C and freezing stress -7°C) was used as a basis of the molecular analysis (see Methods and Materials). Small letters indicate statistically significant differences at P = 0.05.

The difference between T and NT, could be seen most clearly in the results from expression of the gene *ABCG40*, related to the import of ABA in cells. Earlier it was thought, that due to the properties of ABA to pass through the cell membrane in neutral (protonated) form, a special protein transporter was not necessary for its import (Salisbury and Ross, 1985). It was found, however, that such transporter was necessary for the fast response of plants to stress (e.g. stomata closing) (Wilkinson and Davies, 1997). Moreover, mechanism for the initial signal transduction of ABA was found very recently, according to which the import of ABA inside cells is extremely important, because its receptors are located inside the cytoplasm (Park et al., 2009; Ma et al., 2009). Mutant *Arabidopsis* plants in which the expression of the gene *ABCG40* was missing were highly sensitive to drought (Kang et al., 2009). The protein family ABC (ATP-binding cassette transporter) is very large and comprises diverse transporters in all plant taxonomical groups (Verrier et al., 2008). These

proteins are ATP-dependent transporters of the hormones ABA and auxins, but also heavy metals, lipids and other compounds, as well (Rea, 2007). Despite the action of ABA in the stomata closing have been studied in details a long time ago and the role of the secondary messengers such as calcium is well-known (Israelsson et al., 2006), the study of the initial steps of this process has begun only recently. In the stomatal cells of *Arabidopsis*, ABCG40 is synthesized about 8 times more, as compared with the mesophyll cells (Kang et al., 2009). The forementioned, as well as our results clearly showed, that the transport of ABA in the cells by the means of ABCG40 has significance for the response of plants to stress and, particularly, of *A. alpina* to cold stress.

- **qRT-PCR analysis of genes from the metabolism of cytokinins**

10 genes from the metabolism and signal transduction of cytokinins were studied. Three of them were from the gene family IPT (isopentenyladenine transferase - IPT1, 2, 3) from the biosynthesis of cytokinins; four genes from the gene family of CKX (cytoknins oxidase/dehydrogenase - CKX1, 2 ,5, 7), for the degradation of CK; and three genes from the signal transduction of CK – ARR1, 5, 7) (Fig. 32). The transcription of *IPT1* was inhibited very strongly (about 5 times, 2.2 / 0.4) immediately after treatment with 4^{o}C, this inhibition being retained after -7^{o}C in the same degree. The behaviour of the gene *IPT3* was similar, and it should be noted, that it has very low level of expression in NT population. It is known, that both genes participate in the biosynthesis of biologically active CKs from the MEP pathway, with substrates ATP/ADP. In contrast to *IPT1* and 3, in the gene *IPT2* coding for the enzyme tRNA-IPT, responsible for the production of *cis*-zeatin from tRNA (Miyawaki et al., 2006), about two-fold increase in transcription of this gene after treatment with low temperatures (especially at 4^{o}C) was observed. It should be noted, that this increase was observed only in T population, whereas in NT population low temperatures were practically ineffective. Concerning the transcription of genes from the type ARR-a (*ARR1,5,7*), treatment with both types of low temperatures led to inhibition of genes *ARR1,5,7* and a weaker effect upon the gene *ARR1*.

Analysis of the transcription of the genes from the CKX family, coding for the key enzyme CK-oxidase/dehydrogenase, was of particular interest. It is well-known, this is a key enzyme, concerning the content of CKs in plants and their physiological action, respectively. Different response in the behaviour of these genes after treatment with both types of low temperatures in both populations was observed again. Treatment with low temperatures led to stimulation (2-4 fold) of the genes *CKX1* and *CKX2* only in T population, whereas in

population NT change in the expression of the genes was not measured. In contrast to genes *CKX1* and 2, low temperatures led to a strong decrease in the transcription of the genes *CKX5* in both populations and *CKX7*, where change in the transcription of this gene was not measured only in population NT.

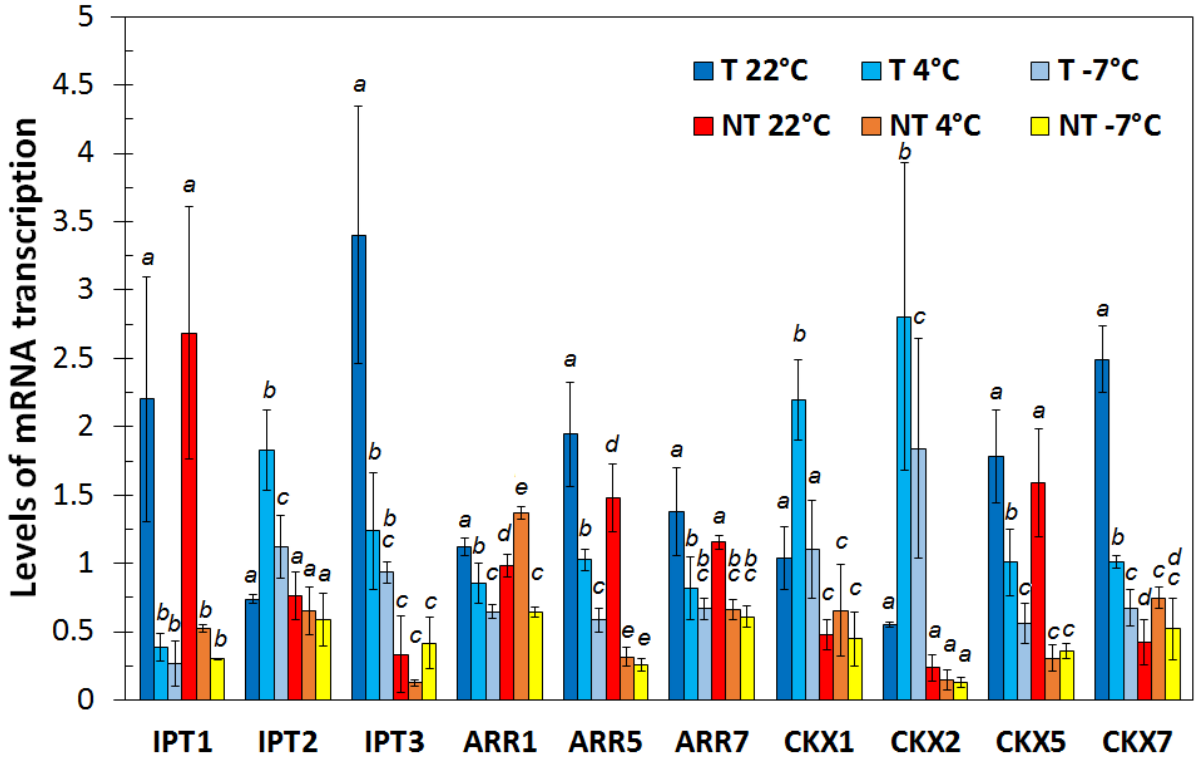


Fig. 32 qRT-PCR transcriptional analysis of genes from the biosynthesis (IPT1, 2, 3), degradation (CKX1, 2, 5, 7) and the signal transduction (ARR1, 5, 7) of the cytokinins. Total RNA sample, isolated from individual plants of the tolerant (T) and non-tolerant (NT) populations of Arabis alpina after the cold stress (chilling +4°C and freezing stress -7°C), was used as a basis of the molecular analysis (see Materials and Methods). Small letters indicate statistically significant differences at P = 0.05.

Summing up the results from the activity of IPT- and CKX-genes in both populations *A. alpina* in conditions of cold stress, it could be said, that their activity correlated with the increase of the ratio *cis*/*trans* zeatins after stress. The genes *IPT1* and *3* are responsible for the synthesis of biologically active cytokinins (Kasahara et al., 2004). They were both inhibited after chilling and freezing stress in both populations. Concerning *IPT3*, this gene had very low transcribtion levels in plants from the NT population. The gene *IPT2* (realizing the biosynthesis of *cis*-zeatins from tRNA) (Miyawaki et al., 2006) was stimulated only in plants from T population after low temperatures, which correlated with the higher content of these

cytokinins in T. Concerning genes, coding for the enzymes from the CKX family, their behaviour correlated with their corresponding substrates, the biologically active cytokinins and *cis*-zeatin, after the cold stress. The biologically active cytokinins are substrates for the enzymes, encoded by the genes *CKX1* and *2*, mostly. In addition, the enzyme CKX1 degrades also the *cis*-zeatin, whereas CKX2 has much lower ability for degradation of *cis*-zeatin (Gajdosova et al., 2011). These genes were stimulated in the tolerant population only. The enzyme, encoded from the gene *CKX5*, one of the other two studied genes from the degradation pathway of cytokinins, degrades the biologically active cytokinins and *cis*-zeatin, but to a lower degree. The last studied gene from the gene family of cytokinin oxidases, *CKX7* codes for an enzyme, degrading the *cis*-zeatins mostly and then the isopentenyladenines (Gajdosova et al., 2011). They both were inhibited in the tolerant population, whereas the CKX7 in NT was not transcribed.

- **qRT-PCR analysis of genes, coding for photosynthetic proteins from PS II and PS I**

qRT-PCR analysis showed, that the chilling at 4°C led to a strong decrease of transcription of studied genes for photosynthetic proteins from PS I in both populations, including the gene for the third protein monomer of the antennae complex of PS I (*LHCAIII*) (Fig. 33). The only exception was the gene psaB for the core protein B of PS I, where both applied low temperatures practically did not exert any effect.

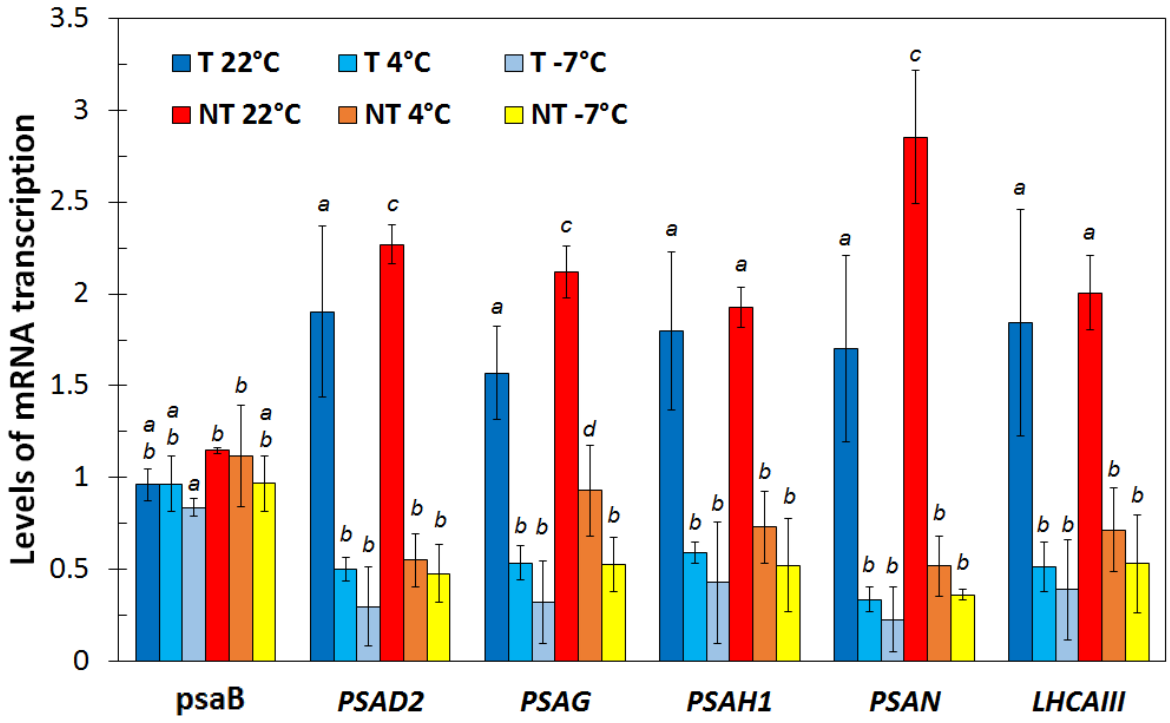


Fig. 33 qRT-PCR analysis of genes, coding for proteins from the reaction centre and the antennae complex of PS I. Total RNA sample, isolated from individual plants of the tolerant (T) and non-tolerant (NT) populations of Arabis alpina after the cold stress (chilling +4°C and freezing stress -7°C), was used as a basis of the molecular analysis (see Materials and Methods). Small letters indicate statistically significant differences at P = 0.05.

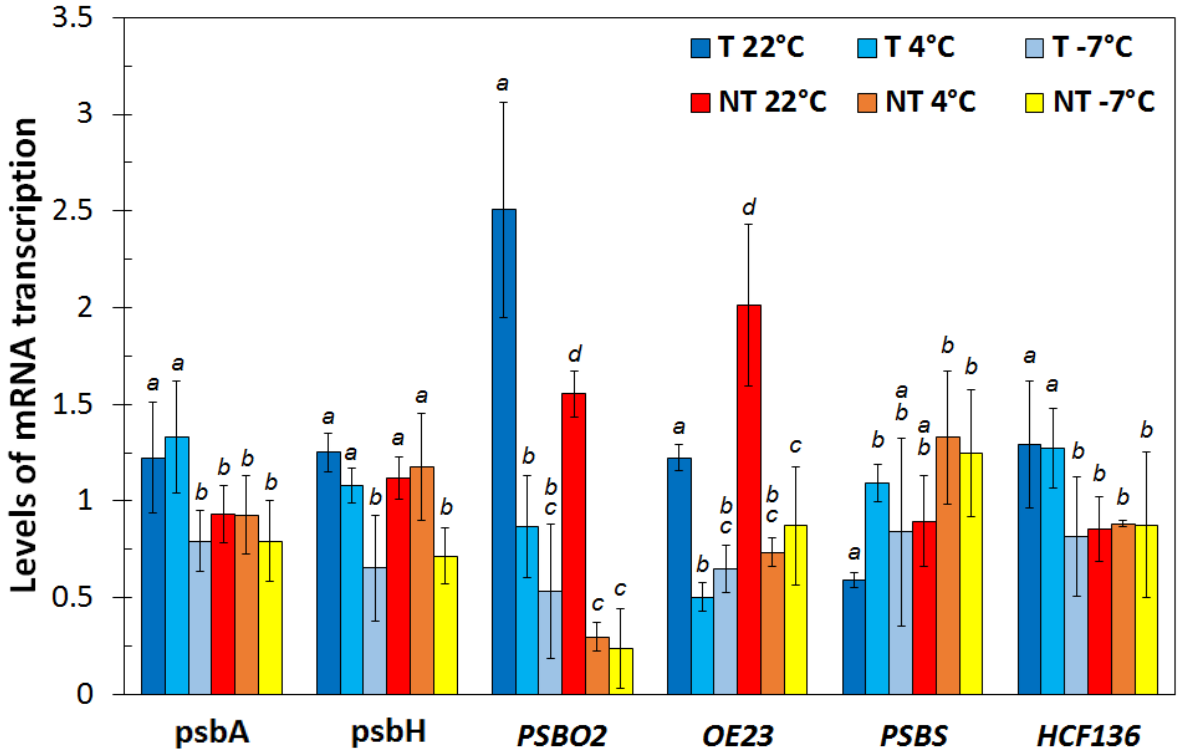


Fig. 34 *qRT-PCR analysis of genes, coding for proteins from the reaction centre, antennae complex and the oxygen-evolving complex of PS II. Total RNA sample, was isolated from individual plants of the tolerant (T) and non-tolerant (NT) populations of Arabis alpina after the cold stress (chilling +4°C and freezing stress -7°C), and was used as a basis of the molecular analysis (see Materials and Methods). Small letters indicate statistically significant differences at P = 0.05.*

The genes for the proteins from PS II showed more complex behaviour after cold stress (Fig. 34). According to their response against low temperatures, they could be separated to three groups: the first group, similarly to the genes for PS I, were strongly inhibited even after 4°C. The genes *PSBO2* and *OE23*, are included here, which code for two of the proteins of the oxygen evolving complex (OEC). The transcription of these genes was inhibited very strongly (60-70%) even after 4°C. The second group of genes (psbA, psbH, *HCF136*) demonstrated high level of tolerance especially after treatment with 4°C, but nevertheless were inhibited after -7°C. The two chloroplast encoded genes psbA and psbH, for the core protein D_1 of the reaction centre of PS II and the side protein H of PS II respectively, as well as the gene HCF136, coding for a protein responsible for the assemble and stability of PS II, are included here. The third group was represented by the gene *PSBS*, which was the only one that showed stimulation after treatment with low temperatures. This nuclear gene codes for

the protein PSBS (CP22), responsible for the non-photochemical quenching of the excessive light energy during light stress.

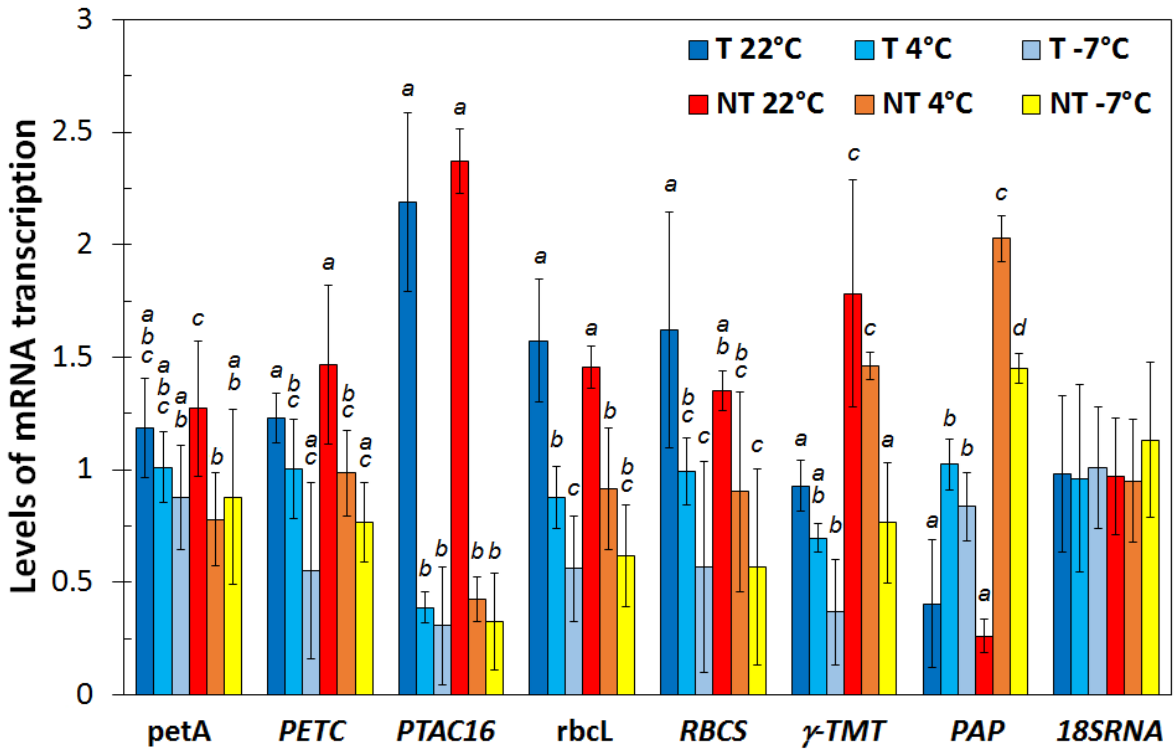


Fig. 35 qRT-PCR analysis of other genes. petA and PETC are genes, coding for the cytochrome f and Rieske protein of the Cytochrome b6f complex, respectively; PTAC16 is a gene for the chloroplast transcription factor; rbcL and RBCS are the genes for the big and small subunits of Rubisco, respectively; γ-TMT (γ-tocopherol methyl transferase) a gene from the biosynthesis of tocopherol α and β; PAP a gene for the chloroplast phospholipid associated (binding) protein. Total RNA sample, was isolated from individual plants of the tolerant (T) and non-tolerant (NT) populations of Arabis alpina after the cold stress (chilling +4°C and freezing stress -7°C), and was used as a basis of the molecular analysis (see Materials and Methods). Small letters indicate statistically significant differences at P = 0.05.

In addition the transcription of other photosynthetic genes (outside these of PS I and PS II), as well as some non-photosynthetic genes was studied. As representatives of the latter we studied the gene *PTAC16* for the chloroplast transcription factor and the genes *γ-TMT* (γ-tocopherol methyl transferase) and *PAP* (phospholipid associated protein), considered as markers for the stress response in plants. The transcription of the additional photosynthetic genes petA and *PETC* from the composition of the Cytochrome b6*f* complex, that code for the proteins cytochrome f and the Rieske protein, respectively, were also studied. Due to their

importance for the production of both Rubisco subunits, the genes rbcL and *RBCS* were studied. Results showed (Fig. 35) that genes from the cytochrome b6f complex were comparatively tolerant to low temperatures, which was shown mainly in T population. In contrast to these two genes, the gene for the chloroplast factor *PTAC16* showed significantly high level of inhibition (around 80%) even after 4ºC which was indication for possible inhibition of the chloroplast transcription even at this temperature. Concerning the genes for the both subunits of Rubisco, they were inhibited to the same degree gradually with the decrease of the temperature in both populations. Transcription of the gene *PAP* in both populations was stimulated by the low temperatures to a higher degree in the NT population. Transcription of the gene *γ-TMT* was inhibited in both populations gradually after treatment with low temperatures.

The study of the expression of genes for photosynthetic proteins from both photosystems showed very high inhibition of the genes for PS I even after chilling (4ºC) in plants from both populations. In contrast to them, the genes for proteins from PS II were not so highly inhibited after the cold stress. The strong inhibition of genes of PS I as compared with the genes of PS II confirmed the higher sensitivity of PS I to low temperatures. Expression of genes for the protein from PS II, PSBS even showed higher activity after the cold stress, which is related to the functions of this protein subunit form PS II. This protein is involved in the quenching of the excessive unused light energy from the photosynthetic apparatus after illumination with light in combination with low temperatures. The protein PSBS is significant for the plant species from the northern latitudes for acclimation to the cold periods of the year (Oquist and Huner, 2003). Thus plants are protected from the unfavourable effects of the light in conditions of low temperatures. The gene for proteins from the oxygen evolving complex (OEC) decreased significantly their expression after chilling (4ºC). Deactivation of the oxygen evolving system is important factor for coping with the free radicals that are generated from photosynthesis in low temperature conditions. Being a source of oxygen that is transformed into reactive oxygen species in conditions of low temperatures due to disruption in the activity of the photooxidising reactions, the inhibition of OEC is an important part from the tolerance to low temperatures of plants. Genes for the proteins of OEC showed very high inhibition even after chilling (the first treatment with low temperatures), indicating unambiguously that its deactivation is an important part from the plant tolerance to low positive temperatures. Genes, coding for proteins from the reaction centre of PS II displayed much lower inhibition than the corresponding genes from the

reaction centre of PS I, demonstrating the significantly higher sensitivity of PS I to low temperatures, as compared with PS II. These results confirm the conclusions, done after the analysis of the photosynthesis by the means of chlorophyll fluorescence, indicating strong inhibition of PS I in both populations even after 4^{o}C and insignificant inhibition of PS II even after -7^{o}C in T.

06. SUMMARY

In the current work plants from two populations of *A. alpina*, from the French Alps and the mountain of Vercors, tolerant and non-tolerant to low temperatures, respectively and plants from Rila mountain (tolerant) were studied. Basic physiological parameters such as electrolyte leakage, as well as photosynthetic intensity by the means of the CO_2 uptake, chlorophyll fluorescence and the modulated 820 nm light reflection were measured. Furthermore, the content of pigments from the photosynthetic apparatus, as well as tocopherols as non-enzymatic antioxidants were analysed. In addition, the content of important phytohormones (ABA, CK, jasmonic acid) was studied and analysis of the transcription rate of genes from the metabolism of phytohormones and photosynthetic proteins was carried out.

Our results showed the content of photosynthetic pigments after cold stress was decreased mostly in the non-tolerant population of *A. alpina* and their content was retained in T. The increase of the pigment from the xanthophyll cycle zeaxanthin to approximately the same degree in both populations after low temperatures was of specific importance. Zeaxanthin is responsible for the non-photochemical quenching of light energy and participates in the protection from abiotic stress (Niyogi et al., 1998). The content of both carotenoid pigments represented with the highest amount, lutein and β-carotene remained unchanged in T, whereas in NT it was strongly decreased even after 4ºC. Lutein is the most important pigment from the composition of the antennae complexes (Standfuss et al., 2005). β-carotene is the second pigment in plant cell and takes part in the content of the thylakoids, stabilizing the properties of membranes (Gruzsecki and Strzalka, 2004).

The content of the non-enzymatic antioxidants, the tocopherols, which protect the photosynthetic apparatus from reactive oxygen species remained unchanged after treatment with low temperatures in both populations except γ- and δ-tocopherol, that were increased only in T population. Analysis of the chlorophyll fluorescence and modulated 820 nm light reflection showed higher sensitivity of PS I, compared with PS II. Transcriptional analysis of mRNA for proteins from the photosystems also demonstrated higher inhibition of the genes for PS I. Analysis of the modulated 820 nm light reflection displayed increase of the reduced compounds after 4ºC, as well as blockage of the electron transport until PS I in NT as compared with T and a stronger inhibition of its functions in NT after minus temperatures. Parameters of the chlorophyll fluorescence are indication for a higher tolerance of the events,

closely related to the activity of the PS II, and the parameters, reflecting events closer to PS I were inhibited more strongly. These results could be a reason to assume, that PS I is more sensitive to low temperatures, as compared with PS II, which bears the larger amount of the cold stress. The analysis of the transcription of genes from both photosystems showed much greater inhibition of the genes from PS I, except the gene for the core protein PsaB, the transcription of which did not change. The gene for the core protein from PS II PsbA, as well as the gene for assembly and stability of PS II *HCF136* also did not change significantly after cold stress in both populations. Both studied genes (*PSBO2* and *OE23*) for proteins from the oxygen evolving complex were inhibited even after 4ºC in both populations. The net photosynthetic activity, as measured by the uptake of CO_2 showed a strong inhibition in both populations (T, NT and Rila) even after 4ºC, which is indication that the dark reactions (phase of the carbon reaction) were inhibited significantly stronger than the light phase. The genes for the small and large subunit of Rubisco were inhibited subsequently after the low temperature treatments in both populations in a way, similar to the net photosynthesis.

The hormonal analysis showed increase in the content of ABA and its degradation products after treatment with low positive temperatures and the high content was retained after -7ºC. The content of the last product from the inactivation of ABA by degradation or the DPA was the highest in T and was practically missing in NT, which possibly was an indicator for the higher metabolic activity of ABA in T. The analysis of the content of the cytokinines /CKs/ displayed two-fold higher concentration of the considered inactive *cis*-zeatins as compared with the biologically active cytokinins. The content of cis-zeatin riboside was the highest, followed by the isopentenyladenin riboside. The behaviour of these phytohormones in both studied populations after low temperatures was similar and retained unchanged after the low temperatures in T and was decreased after 4ºC and more strongly after -7ºC in NT. The higher content of *cis*-zeatins was a reason to assume that these cytokinins have a high biological activity in *A. alpina*.

The transcription levels of genes from the metabolism of ABA and cytokinins showed correlation with the content of these phytohormones. Genes from the biosynthetic path of ABA displayed activation after cold stress. The gene for import of ABA inside cell was activated especially strongly in T after low temperatures, in contrast to NT. This might be a reason for ABA to have signal function except stress function in the T population. Concerning the genes for synthesis of biologically active cytokinins (*IPT1* and *3*) (Kasahara et al., 2004) they were inhibited after low temperatures in both populations, and *IPT3* was not represented

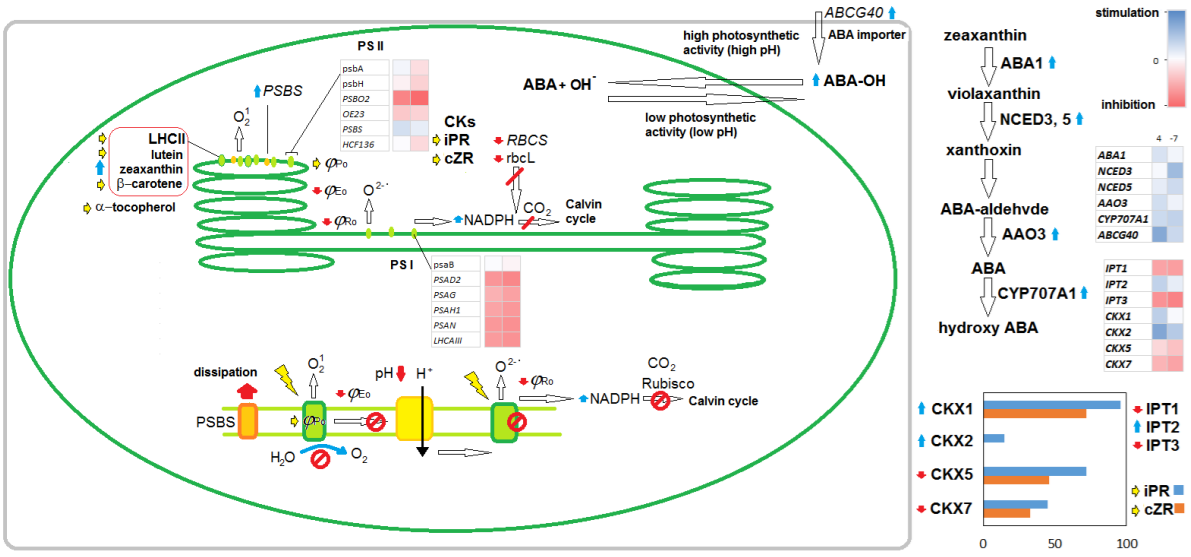
in plants from the population NT. The gene *IPT2* (realizing the biosynthesis of *cis*-zeatins from tRNA) (Miyawaki et al., 2006) was stimulated after low temperatures only in plants from population T, and correlated with the higher content of these cytokinins in T. Enzymes, encoded from the genes *CKX1* and *2* have the biologically active cytokinins for substrates, and *CKX1* also and the *cis*-zeatin (Gajdosova et al., 2011). These genes were stimulated in the tolerant population only. *CKX5*, one of the other two studied genes from the degradation pathway of cytokinins codes for an enzyme, degrading the biologically active cytokinins and *cis*-zeatin. The enzyme encoded from the last studied gene of the gene family, *CKX7* degrades the *cis*-zeatins to a highest degree and next the isopentenyladenines (Gajdosova et al., 2011). These genes were inhibited in the tolerant population, and the CKX7 in NT was not transcribed.

It is known, that the process photosynthesis mostly is affected by abiotic stress, including low temperatures (Theocharis et al., 2012). Photosynthesis is the main anabolic process in plants, and except that it is a source of reactive oxygen species (Apel and Hirt, 2004). In conditions of stress from low temperatures, higher amounts of reactive oxygen species are being produced, due to the damages in plant cell and mainly the photosynthetic apparatus (Krieger-Liszkay, 2004). Initially, Rubisco is inactivated and the CO_2 uptake is inhibited, due to which reduced NADPH accumulates. When higher amount of reduced compounds are accumulated in the electron-transport chains, electrons donated from the photosystems do not go for reduction of NADPH, but bind to oxygen and instead reactive oxygen species form (Szarka et al., 2012). The ability for neutralization of these reactive oxygen species by carotenoid pigments or antioxidant activity could be a possible reason for the resistance of the tolerant plant populations (Jahns and Holzwarth, 2012). Because cytokinins are regulators of the photosynthetic apparatus, as well the activity of Rubisco (Lerbs et al., 1984), the characteristic retain of the content of cytokinins in T probably is responsible for the preservation of the photosynthetic activity in plants from this population after cold stress. Activation of specific genes from the biosynthesis of cytokinins such as *IPT2* for synthesis of cis-zeatins was also higher in T population. The activation of certain protector mechanisms against cold stress in T as well as in NT was notable. For example, after treatment with low temperatures the xanthophyll cycle in plants from both populations was activated. Furthermore, the content of α-tocopherol, having the highest content amongst the tocopherols remained constant. Besides that, specific genes for photoprotectors such as PSBS

were also activated in both populations in the same level, as well as genes from the biosynthesis of ABA.

Being the most important process for the metabolic activity of plants, photosynthesis is affected to the highest degree from stress, including low temperatures. Photosynthesis is a highly energetic process, where high amount of reactive oxygen species form. After cold stress, the generation of these reactive oxygen species is more and more difficult to overcome by plants and they inflict multiple damages on the components of the cell and the photosynthetic apparatus. Brestic et al., 2013 showed that ABA could regulate positively the tolerance of PS II by the means of increasing the antioxidant activity. Transgenic tobacco with IPT regulated by stress promoter of the aging displayed higher tolerance to drought, due to the increase of the photorespiration regulated by the cytokinins (Rivero et al., 2009).

A GENERAL SCHEME FOR THE PHYSIOLOGICAL, BIOCHEMICAL AND THE MOLECULAR PROCESSES INSIDE PLANTS FROM THE TOLERANT POPULATION OF *A. ALPINA* AFTER COLD STRESS.



Blue arrows show stimulation, red ones show inhibition and the yellow show lack of change.

07. CONCLUSIONS

1. Two tolerant to minus temperatures (-7^{o}C) populations of *A. alpina* were chosen, from the French Alps (T) and Rila mountain, respectively, as well as one non-tolerant to this stress population (NT) from the mountain of Vercors, France.

2. The low positive (4^{o}C, chilling stress) and negative temperatures (-7^{o}C, freezing stress) inhibited the net photosynthetic activity in the three populations of *A. alpina*, averagely with 55% and 75%, the negative effect being the strongest in the NT population respectively. Only the plants from populatioins T and from Rila mountain recovered their net photosynthesis after the minus stress.

3. A decrease in the chlorophyll pigments (more specifically of chlorophyll *a*) was determined under the influence of both types of low temperatures in both populations T and NT, this decrease being more clearly seen in NT. In contrast to the chlorophylls, the carotenoid pigments decreased after cold stress only in NT population, whereas in T population their content remained constant. Inside the frames of the xanthophyll cycle an increase of zeaxanthin and decrease of violaxanthin (de-epoxygenation of violaxanthin) was determined.

4. As compared with φ_{Po}, parameters of the prompt chlorophyll fluorescence φ_{Eo}, φ_{Ro} and PI_{total} were inhibited to a higher degree in both types cold stress, and inhibition at -7^{o}C was stronger in NT population. Considering these parameters, plants from Rila mountain demonstrated higher tolerance to the cold stress as compared with T population.

5. MR_{820}, as a measure for the activity of PS I was inhibited to a higher degree in population NT. In contrast to population T, after treatment with minus temperatures, the activity of PS I was not recovered only in population NT. Plants *A. alpina* from Rila mountain showed higher degree of tolerance to both types of cold stress by this parameter, as well.

6. In contrast to the genes for PS II both types of low temperatures (4^{o}C and -7^{o}C) inhibited to a higher degree in both populations T and NT the transcription of the studied genes for proteins from PS I (*PSAD2, PSAG, PSAH, PSAN* and *LHCAIII*). Transcription of genes from PS II (*psbA, psbH* and *HCF136*) was comparatively

tolerant to treatment with low temperatures except *PSBO2* and *OE23*. The only gene from PS II, stimulated after cold stress was *PSBS*.

7. Genes from the Calvin cycle for the small (*RBCS*) and large (rbcL) subunits of Rubisco were inhibited to the same degree by both types of cold stress in both studied populations T and NT of *A. alpina*.

8. Increase of ABA and its oxidized products PA and DPA was determined after cold stress (4^{o}C), as well as retain of their content after -7^{o}C in both studied populations T and NT. Correspondingly to the increased content of ABA and its catabolites, both types cold stress resulted increase of the transcription of the genes from the biosynthesis (*ABA1, NCED3, NCED5, AAO3*), inactivation (*CYP707A1*) and transport of ABA (*ABCG40*).

9. In contrast to NT population, treatment with 4^{o}C, as well as with -7^{o}C did not change content of biologically active cytokinins (mostly iPR) and the cytokinins from the *cis*-zeatin type (mostly *cis*-ZR). Both types cold stress caused inhibition of the transcription of genes *IPT1* and *IPT3* for biosynthesis of biologically active cytokinins in T and NT, whereas the transcription of the gene *IPT2* for biosynthesis of *cis*-zeatins was increased only in T, which correlated to the higher amount of *cis*-zeatins in this population. Stimulation of genes from the CKX family (mainly *CKX1* and *CKX2*) was determined under the effect of low temperatures in T population.

08. CONTRIBUTIONS

1. After a period of acclimation to low positive temperatures (4^{o}C) a tolerant to minus temperatures (-7^{o}C) population of *Arabis alpina* L. with areal Rila mountain, "Seven Rila Lakes" was determined. This population was similar to already known tolerant population T of *A. alpina* from the French Alps. Both tolerant populations were differentiated from the population NT of *A. alpina* from the mountain Vercors (France), being non-tolerant to minus temperature.

2. It was shown for the first time, that in contrast to the non-tolerant population NT, tolerant to frost populations T of *A. alpina* from the French Alps and Rila mountain could recover the intensity of their net photosynthesis after treatment with -7^{o}C again by transition in a period of acclimation at 4^{o}C.

3. The comparative analysis between populations T and NT of *A. alpina* showed that in contrast to NT, where both types cold stress at 4^{o}C and -7^{o}C lead to strong inhibition mostly of chlorophyll *a* and the main carotenoid pigments lutein and β-carotene, in tolerant T population their content remained relatively constant. On the background of similar changes in the pigments from the xanthophyll cycle (increase of zeaxanthin on the account of violaxanthin) in both populations, the preserved content of lutein and β-carotene in T population could be related to the acquisition of the tolerance to minus stress.

4. The complex comparative analysis by the means of chlorophyll fluorescence and the transcriptional (qRT-PCR) analysis of genes for proteins from PS I and PS II displayed higher sensitivity of PS I to both types of cold stress. Parameters of the chlorophyll fluorescence and the transcription of genes from PS II (*psbA*, *psbH* and *HCF136*) were relatively tolerant to treatment with low temperatures only in population T, and the gene for the protein PSBS was even stimulated at -7^{o}C.

5. For the first time in two different to their tolerance to minus temperatures (-7^{o}C) populations of *A. alpina* was carried out complex analysis including determination the content of ABA and all types of cytokinins, as well as the transcription of main genes from their metabolism and signal transduction. Retain in levels of the main biologically active cytokinin iPR together with the *cis*-ZR was determined only in population T, whereas ABA and its oxidizing forms PA and DPA were retained in both populations T and NT. Changes in the content of ABA and cytokinins were correlated with the rate of transcription of genes from

their biosynthesis, degradation and transport (*ABA1, NCED3, NCED5, AAO3, CYP707A1* and *ABCG40*) for ABA and *IPT1, IPT3, IPT2, CKX1* and *CKX2* for CK, respectively.

6. Analysis of main parameters of photosynthesis, together with the content of key hormones and the expression of genes from their metabolism, gived us the base to assume, that the tolerance to cold stress (-7^{o}C) of the population T of *A. alpina* from the French Alps could be due to a complex of factors, including high levels of chlorophylls and carotenoid pigments, incl. those from the xanthophyll cycle, higher activity of the electron transport in PS II and higher content of biologically active and *cis*-forms of the cytokinins as main regulators of the process photosynthesis.

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