Main points of the PhD thesis

Changes in gene expression and metabolic processes during stress adaptation in plants

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Introduction

As a consequence of global climate change, extreme values of annual temperatures or rainfall distribution are becoming increasingly frequent, both in Hungary and elsewhere. These rapid changes in environmental factors induce new adaptation processes in plants. The morphological, genetic and physiological changes occurring as a result of these processes may have an unfavourable effect on the quality and quantity of crops, raising new challenges for plant breeders and growers alike.

In the course of the PhD research, the aim was to acquire a better understanding of the role of cold acclimation and light intensity in the development of frost tolerance. Wheat (*Triticum aestivum* L.) is one of the most important cereals in Hungary and throughout the developed world. *Arabidopsis thaliana*, on the other hand, is a model plant with known genetic background, widely used in biological research. The cultivars, mutants and transgenic lines of this species can be used for the investigation of a range of molecular and physiological problems.

The focus in the present work was on the genetic background of stress responses in wheat. The results of previous experimentation showed that the light intensity during the cold hardening of winter wheat is of outstanding importance for the development of satisfactory cold tolerance. Following the physiological analyses, attention was turned to differences in gene expression during cold hardening as a function of the genotype (spring or winter wheat varieties) and light intensity. After completing the microarray analysis, answers were sought to the following questions: **(I) How does light intensity influence gene expression in wheat during cold hardening? Which genes exhibit a significant change in expression in response to cold and various levels of illumination? (II) What protective compounds are responsible for the development of a satisfactory level of frost tolerance in wheat?**

In *Arabidopsis* investigations were made on the changes occurring in the antioxidant, hormonal and other metabolic defence pathways during cold hardening. In the first place freezing tests were performed to determine **(III) whether there were any differences in cold tolerance between ascorbate- and salicylic acid-deficient *Arabidopsis* genotypes**. The next step was to investigate **(IV) how the frost defence strategies of *Arabidopsis* genotypes changed at different temperatures and which physiological factors could be responsible for improved levels of frost tolerance**.

A similar experimental system was used to examine the recently re-discovered *Arabidopsis* mutant *Atnoa1* (nitric oxide associated), the physiological background of which

is known in less detail. After testing *noa1* mutants for frost tolerance, the following question arose: **(V) What anti-stress pathways are responsible for the development of satisfactory frost tolerance?**

The last part of the work was aimed at investigating the effect of temperature and light quality on the polymorphism of the shade avoidance syndrome in *Arabidopsis* introgression lines. The question raised was: **(VI) Which genes are responsible for the development of the shade avoidance syndrome?**

Materials and methods

Plants of winter (*Triticum aestivum* L. var. Mv Emese) and spring (*T. aestivum* L. var. Nadro) wheat were used for the gene expression analysis. The seeds were sown in a 3:1 mixture of soil and sand and the plants were raised in soil at 20°C in the phytotron of the Agricultural Institute in Martonvásár. The light intensity (PPFD) was 250 µmol m^{-2} s^{-1} (NL). Cold hardening was carried out for 12 days at 5°C at NL or at lower light intensity (LL, PPFD=20 µmol m^{-2} s^{-1}).

The *Arabidopsis thaliana* genotypes used for the physiological analyses were the wild type Columbia (Col-0), four mutants: the ascorbate (AA)-deficient *vtc2-1* (Conklin et al. 2000), *eds5*, which has a low SA level (Nawrath et al. 2002), *sid2*, which has deficient SA synthesis (Wildermuth et al. 2001) and *noa1*, an NO-deficient mitochondrial cGTPase mutant (Moreau et al. 2008), and two transgenic lines, GLOase: L-gulono-1,4-lactone oxidase, which, when transformed into *vtc*, causes its complementation (Radzio et al. 2003), and NahG, which carries the transgene of an enzyme that decomposes SA, converting it into catechol. The seeds were germinated in soil at 23°C for 3 weeks with short-day illumination at PPFD = 50 µmol m^{-2} s^{-1}. The plants were then grown at 21°C with long-day illumination at PPFD = 150 µmol m^{-2} s^{-1}. Cold hardening took place from the 8^{th} week at 4°C for 4 days at PPFD= 90 µmol m^{-2} s^{-1}. Freezing tests were carried out at –9 and –11°C in the dark for 24 h. Surviving plants were counted 21 days after the end of the freezing treatment.

The QTL mapping was performed on NIL2–8 plants with an L*er* genetic background and a Cvi introgression (Keurentjes et al. 2007). The seeds were germinated at 4°C in the dark on Lehle medium , after which the plants were grown at 21°C with long-day illumination at a light intensity of PPFD=130 µmol m^{-2} s^{-1}. In order to observe the polymorphism of shade avoidance, illumination at PPFD=100 µmol m^{-2} s^{-1} with a low red/far red (R/FR) ratio was

applied from the 3rd week at a temperature of 16°C. The photomorphogenetic changes were evaluated visually.

The isolation of RNA for gene expression analysis was carried out using TRIzol reagent (Invitrogen). The amplification and labelling of the RNA was performed after hybridisation onto an Agilent 4X44K Wheat Chip, following the instructions of the manufacturer. The primary analysis of the data was carried out using the linear model of the ImFit and eBayes functions of LIMMA (Smyth 2004), with p values adjusted to the testing method of Benjamini and Hochberg (1995). The downloaded annotation of the Agilent chip proved to be incomplete, so the genes were classified into gene ontology (GO) categories using re-annotation data.

DNA extraction for the mapping of the SAS QTL in the L*er*(Cvi) NILs was performed on 96 well plates with the addition of ice-cold isopropanol. Genotyping in the 12.65 and 11.61 Mb regions of chromosome 2 was carried out using two allele-specific In/Del markers and in the 11.16 Mb region with an SMP marker.

For the physiological analyses, the quantum efficiency parameters F_v/F_m and $\Delta F/F_m'$ were measured using a PAM-2000 instrument (Walz, Effeltrich, Germany). The plants were dark-adapted for 30 min before the measurement of F_v/F_m. The relative chlorophyll content was determined on the third fully developed leaf using a CL-01 chlorophyll meter (Hansatech Instruments Ltd., Norfolk, UK).

Frozen leaf samples were shaken in distilled water, after which the electrolyte leakage was measured on the basis of conductance using an Automatic Seed Analyzer (ASA610, Agro Science).

Proline was determined on the basis of the ninhydrin reaction (Bates et al. 1973), while the gallic acid equivalence method was used as described by Salluca et al. (2008) to determine polyphenols.

For the measurement of ascorbate (AA) the samples were homogenised in metaphosphoric acid, and the total AA content was determined by means of dithiothreitol reduction, followed by measurement using an isocratic Waters 2690 HPLC instrument. The quantity of the oxidised form was obtained as the difference between the total and reduced forms.

The antioxidant enzymes were isolated according to Janda et al. (1999) and measured using a Shimadzu UV-VIS 160A spectrophotometer. The glutathione reductase (GR) activity was recorded by measuring the reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, as described by Smith et al. (1988). The activity of ascorbate peroxidase (APX) was

determined by monitoring the consumption of AA at 290 nm, while catalase (CAT) activity was recorded at 240 nm as the decrease in the hydrogen peroxide quantity (Janda et al. 1999). In the case of guaiacol peroxidase (GPX), activity was detected spectrophotometrically as the increase in absorbance caused by the oxidation of guaiacol (Ádám et al. 1995).

The extraction and measurement of monodehydro-ascorbate reductase (MDHAR) was performed as suggested by Krivosheeva et al. (1996), by detecting the conversion of dehydro-AA to AA at 265 nm.

The HPLC analysis of salicylic acid was performed using the method reported by Meuwly and Métraux (1993) after extraction with methanol. Ortho-anisinic acid was used as an internal standard and para-hydroxybenzoic acid as the extraction carrier. The measurements were performed on a Waters HPLC instrument.

Polyamines were determined by HPLC (Waters) after extracting the samples with perchloric acid, followed by derivatisation with dansyl chloride according to Smith and Davies (1985).

Samples for ACC and MACC measurements were prepared as described by Tari and Nagy (1994), with some modifications, and ACC was measured by gas chromatography after conversion to ethylene, using the method of Lizada and Yang (1979). The MACC content was calculated as the difference in the ACC content before and after hydrolysis.

The majority of the results are the means of 5×3 measurements. When this was not the case, special mention is made of the fact in the relevant section of the thesis. Significance was determined using Student's two-sample *t*-test, while principal component analysis (PCA) was used for similarity analysis.

Results and conclusions

1. The results of global gene expression analysis revealed the extent to which the activity of the genes changed in response to cold and light intensity in the individual varieties. On the basis of two varieties and two levels of light intensity, four comparisons were made: Mv Emese at *normal light intensity (NL)* vs Nadro NL, Mv Emese at *low light intensity (LL)* vs Nadro LL, Mv Emese NL vs Mv Emese LL, and Nadro NL vs Nadro LL.

Within each comparison selection was made for genes with an at least two-fold change (FC) compared with the control. In response to low light intensity a rise in activity was observed for 97 genes in the winter variety Mv Emese and for 106 genes in the spring variety Nadro. In the same way, when comparison was made on the basis of different levels of light

intensity, 387 genes exhibited reduced activity at low light intensity in the winter wheat and 243 in the spring variety. These included the general stress proteins (CBF transcription factors, LEA proteins, dehydrins, low temperature-induced proteins), enzymes involved in the synthesis and integrity of membranes (phosphoethanolamine methyltransferase) and enzymes responsible for the synthesis of various hormones involved in protection against cold stress (aldehyde oxidase2, 1-aminocyclopropane-1-carboxylate oxidase) or for the synthesis of certain osmotic agents (fructan 1-fructosyltransferase). The products of other genes have a direct or indirect effect on photosynthesis (pheophorbide *a* oxygenase, chlorophyll-binding proteins, cytochrome P450). Little overlapping was found when the gene expression lists of the varieties were compared at different light intensities. One such gene was that coding for ACC oxidase, a key enzyme in ethylene synthesis; another was the glutathione-S-transferase gene. These exhibited illumination dependence for both varieties. The expression of several stress-related genes (coding for cold acclimation protein, dehydrins, CBF transcription factors) was higher at normal light intensity in Mv Emese than in Nadro.

When the genes exhibiting different levels of expression in Mv Emese and Nadro at normal light intensity were compared with those where differences were detected in Mv Emese under normal and low light conditions, it was possible to detect a light intensity- and variety (Nadro)-dependent effect, demonstrating that some genes (e.g. aldehyde oxidase2) had decreased activity in Nadro at normal light intensity and in Mv Emese at low light intensity.

When the gene expression list for Nadro under normal vs low light intensity was compared with that for Mv Emese vs Nadro at normal light intensity, expression was found to be regulated in a variety- and light intensity-dependent manner. At normal light intensity the expression of the genes for glutathione-S-transferase, cytochrome P450 and lipoxygenase was found to be greater in Nadro than in Mv Emese, while these were suppressed at low light intensity.

A comparison of the gene lists for the individual treatments demonstrated that normal light intensity had a substantial effect on gene expression during cold hardening, but further experiments will be required to obtain more precise knowledge on the changes in expression in various treatments and varieties.

2. Among the osmoprotectants, the level of proline was found to be influenced by light intensity during cold hardening in wheat. At NL a transitional peak was observed in both varieties on the 3rd day of cold hardening. On the 7th day and on the last day tested, the 12th day, the proline level was lower than on the 3rd day, but these values were still significantly

higher than the levels recorded at the control temperature. In the case of low light intensity, no changes could be detected in either variety.

The total content of phenolic compounds was measured daily during the first six days of cold hardening. In both wheat varieties a transitional reduction was observed up to the 3rd day of treatment at both LL and NL, followed by a slow but continuous rise. This trend was similar for both varieties and both treatments. In the case of Nadro a significantly higher total phenol content was detected on the last day at both light intensities compared with the control (at the growth temperature prior to cold hardening), while in the winter wheat variety, Mv Emese, this was only true for NL.

3. The survival of the *Arabidopsis* mutants and transgenic lines was evaluated 21 days after freezing. The survival rate was 40–50% for plants treated at –9°C, with the lowest rate for GLOase plants and the highest for *sid2* plants. At –11°C the number of surviving plants was considerably lower. None of the GLOase, *eds5* or NahG plants survived, while the survival rate was below 20% for Col-0 and *sid2*. Only a very small number of *vtc2-1* plants survived. The differences in the freezing survival rates proved that mutant and transgenic variants with diverse physiological traits exhibited substantial variation in frost tolerance.

4. Investigations on the physiological background of differences in frost tolerance revealed that the change in the chlorophyll content in response to cold hardening could have an impact on quantum efficiency parameters, the most pronounced reduction being observed for $\Delta F/F_m'$. Several genotypes exhibited similar changes in parameters related to photosynthesis. A comparison of antioxidant enzyme activity at control and hardening temperatures showed a significant rise in GR activity in all the genotypes. No rise in activity could be observed for MDHAR, while that of APX only increased in the case of *eds5* and *sid2*. The quantity of AA forms generally decreased in the Col-0 and *vtc2-1* genotypes. A slight reduction in the SA quantity was observed in SA-deficient mutants and in the NahG transgenic line. In response to cold hardening, the free form of SA declined significantly in the *vtc2-1* and GLOase, while it rose in NahG, compared with the values recorded at the control temperature. At both temperatures the ratio of the bound form of SA varied considerably between the genotypes, decreasing significantly in *vtc2-1* and *eds5* in response to cold hardening. A reduction was also observed for Col-0, while GLOase, *sid2* and NahG exhibited a slight rise. Among the polyamines (PA) the level of putrescine increased significantly in response to cold hardening, while that of spermine dropped substantially. When differences between the genotypes were investigated, no significant differences were found at either the control or the hardening temperatures. The changes in the quantities of PAs

suggest that the levels of putrescine and spermine are strongly influenced by cold. The substantial increase in the putrescine content could have been due to a temporary accumulation, while the formation of spermine may be a slower process. No significant difference in the ACC level could be detected between control and cold-hardened plants. A slight increase in ACC content was observed in Col-0 and *vtc2-1* and a slight decrease in GLOase. In response to cold hardening, there was a slight reduction in the MACC content in Col-0, *vtc2-1* and NahG and an increase in GLOase. In *sid2* there was a significant reduction in the MACC level.

5. Unhardened *Atnoa1* plants were much more sensitive to frost than the cold-hardened plants, with greater damage to the cell membrane, resulting in a disturbance in the ion equilibrium. The *noa1* variant contained less chlorophyll than Col-0. The F_v/F_m and $\Delta F/F_m$' parameters had significantly lower values in *noa1* plants than in the wild genotypes. Among the antioxidant enzymes, the GR activity was significantly higher at the hardening temperature in the mutant than in Col-0. The mutant had significantly higher levels of free and bound SA in both control and cold-hardened plants. Among the polyamines there was no difference between the two genotypes in the putrescine level either at control or at hardening temperature, but in both varieties cold hardening caused a significant increase. The quantity of spermine declined significantly in response to cold in both genotypes. A difference between the genotypes was only found at the control temperature, where the spermine level was significantly higher in *noa1*.

6. The polymorphism of the shade avoidance syndrome (SAS) was shown in the experiments to be temperature-dependent. Earlier observations revealed that the plants responded with longer leaves and petioles to low R/FR illumination at temperatures below 20°C (SAS1 strategy). In the present work, petiole elongation could be observed from the 10^{th} day when low R/FR illumination was applied at 16°C. Good agreement was observed between the physiological observations and genotyping in the F_2 and F_3 generations. Mapping revealed that the SAS QTL was located in the 11.2 MB region on chromosome 2. This region also contains the Erecta (ER) and Erecta-like (ERL2, ERL2) genes, which play a general, complex role in photomorphogenetic processes. The participation of these genes in the shade avoidance syndrome was confirmed by the present results.

Conclusions that can be drawn from the results

(I) Global gene expression analysis on wheat proved that light intensity is an important factor during the cold period. Genes involved in the development of frost tolerance, exhibiting a significant change in expression, were identified, some of which were related to photosynthetic processes and protection against frost, and others to the glutathione or hormone metabolism.

(II) In both winter and spring wheat the proline level at normal illumination was found to be higher throughout the cold hardening period than before hardening. At low light intensity, however, no significant difference was found for either variety.

(III) The frost tolerance of ascorbate- and salicylic acid-deficient mutant and transgenic variants of *Arabidopsis* proved to be extremely varied. Down to a critical sub-zero temperature, shown in this work to be –10°C, the plants were capable of regeneration.

(IV) Among the physiological variables examined, the role of antioxidant enzymes, ascorbate and salicylic acid in defence against the damaging effects of cold and in the development of satisfactory frost tolerance was found to be less important than that of polyamines.

(V) Investigations on the cold resistance of *Atnoa1* mutants revealed the importance of photoprotective processes and the role of salicylic acid and polyamines.

(VI) In *Arabidopsis* a QTL responsible for shade avoidance under low temperature conditions (SAS1) was mapped in the 11.2 MB region of chromosome 2. The Erecta (ER) and Erecta-like (ERL1, ERL2) genes were identified as candidate genes.

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List of papers forming the basis of the thesis

A Papers published in peer-reviewed journals

Majláth I, Szalai G, Soós V, Sebestyén E, Balázs E, Vanková R, Dobrev PI, Tandori J, Janda T (2012): Effect of light on the gene expression and hormonal status of winter and spring wheat plants during cold hardening. Physiol. Plant. 145/2: 296–314. **IF: 3.112**

Majláth I, Szalai G, Vankova R, Papp I, Janda T (2011): At*noa1* mutant Arabidopsis plants induce compensation mechanisms to reduce the negative effects of the mutation. J. Plant Physiol. 168: 1184–1190. **IF: 2.791**

Szalai G, Horgosi Sz, Soós V, **Majláth I**, Balázs E, Janda T (2011): Salicylic acid treatment of pea seeds induces its de novo synthesis. J. Plant Physiol. 168: 213–219. **IF: 2.791**

B Abstracts in conference proceedings

Majláth I, Szalai G, Janda T (2012): Exploration of cold signalling related to ascorbate and salicylic acid in Arabidopsis thaliana. XVIII. Congress of the Federation of European Societies of Plant Biology, Freiburg 29 July-3 August 2012 p. 372.

Majláth I, Szalai G, Papp I, Vanková R, Janda T (2011): At*noa1* mutation may induce temperature acclimation mechanisms in Arabidopsis thaliana. Acta Biologica Szegediensis 55/1: 113–115.

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