SZENT ISTVÁN UNIVERSITY

INVESTIGATION OF THE ABIOTIC STRESS TOLERANCE AND GRAIN WEIGHT DETERMINING CANDIDATE GENES VIA STABLE GENETIC BARLEY TRANSFORMATIONS

PhD thesis

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BACKGROUND AND OBJECTIVES

The understanding of the plants' defence mechanisms to the environmental stresses is fundamental for the maintainance of the crop safety in extreme environmental conditions.

The isolation of the prosperous allel variants and their application by crossing could be one of the most important resources in the breeding for drought tolerance. The reliable designation of the genotypes' drought tolerance is a key step in the clarification of the candidate genes' role in the resistance. Studies proved that in certain crop plants the gene expression of the aldo-keto reductases is different in stress tolerant and sensitive cultivars. In our experiments we tried to find a similar pattern, and also tried to determine the genomic background of variable tolerance levels of the cultivars. In connection, we investigated the role of two barley genes (*HvARH1* and *HvSRG6*) in the adaptation to drought by gene expression analysis in different barley genotypes.

The dissertation's next part presents the results of the characterization of transgenic barley plants generated by *Agrobacterium*-mediated plant tarnsformation. The investigated gene was the *MsALR* (aldose reductase isolated from *Medicago sativa*) which can protect the cells against stresses by the reduction of the reactive aldehydes. It was previously shown, that transgenic plants overexpressing *Medicago sativa* aldo-keto reductases exhibited higher tolerance against different abiotic stresses such as drought (Oberschall et al. 2000), UV (Hideg et al. 2003) and heat (Turóczy et al. 2011). In our experiments we determined the level of the protective impact, and also we investigated wether the detoxification of these reactive compounds is more important in the cytosol or in the chloroplast. For this purpose we generated a gene construct that allows the transport of this protective protein to the chloroplast. This candidate gene was overexpressed in transgenic barley plants and the effect of the elevated expression was investigated during abiotic stress conditions (dehydration, salt, and carbonyl). The characterization was based on a transient expression test system (Marzin et al. 2008).

Beside of the production of abiotic stress resistant plants, another important aim was to increase the potential of the crop production; the enlargment of the seeds and the fostering of the grain fill is also a relevant aspect. Based on the literature we selected one gene that can influence the grain size in barley. This gene is the *GW2* homologue (grain weight 2 gene that plays role in the grains' size determination) that was isolated and characterized in rice. It was described that the antisens orientation of this gene caused an increase in the grains' width and length in rice (Song et al 2007). In our studies we investigated whether the silencing of this gene's homologue in barley leads to similar results.

The main aims of the dissertation:

1. The analysis of the *HvARH1* and *HvSRG6* genes (which may play role in drought tolerance) expression during absiscic acid (ABA) treatment.

2. The adaptation of the genetic transformation of barley via *Agrobacterium*-mediated transformation.

3. Construction of expression cassettes using the *MsALR* gene targeting this gene to the cytoplasm (p6d35S-MsALR) and to the chloroplasts (p6d35S-cpMsALR), respectively. Verification of the transgene's integration, and the determination of the copy number using molecular methods.

4. Investigation of the *MsALR* gene's role in abiotic stress tolerance based on a fluorescence detection method using the generated transgenic lines suffering salt, dehydration and carbonyl stress treaments. Confirmation of the tolerance gained from the fluoresence ratio data by biochemical techniques (chlorophyll content, proline and thiobarbituric acid accumulation measurements).

5. Generation of transgenic barley lines harbouring the *GW2* homologue in antisens orientation and the characterization of these lines using molecular methods. Phenotyping of these lines by application of a pixel-based process for the grain size determination.

MATERIALS AND METHODS

Plant growth and materials

The stable barley transformation experiments were carried out on immature barley embryos (*Hordeum vulgare* cv. 'Golden Promise').
For the transient assay system segments of five primary leaves (with a length of 5-6 cm) were detached from 10 days old transgenic and non-transgenic (GP) barley seedlings and placed onto 0.5% (w/v) water–phytoagar (Duchefa).

Barley transformation

The stable transformation of immature barley embryos (*Hordeum vulgare* L. 'Golden Promise', GP) was accomplished as described (Hensel et al. 2004) using the LBA4404 *Agrobacterium tumefaciens* strain.

Quantitative RT-PCR measurement of *MsALR* gene expression

The cDNAs for the Quantitative-PCR reactions were obtained from the total RNA isolated from young barley leaves according to the AGPC (acid guanidinium thiocyanate-phenol chloroform) method (Chomczynski and Sacchi, 1987). The qRT-PCR reactions were carried out in the ABI PRISM 7000 Sequence Detection System. The $2^{-\Delta\Delta Ct}$ C(T)) method was used to analyse the real-time PCR data (Livak and Schmittgen 2001).

Transient expression of fluorescent proteins by microprojectile bombardment

For the microprojectile bombardement, gold particles (diameter 1 μm) were coated with two different plasmid DNA (pGFP and pUbi-DsRed-nos) as described by Róbert Mihály (2009).

The transient assay vectors were provided by Dr. Patrick Schweizer (IPK Gatersleben, Genome Analyses Department, Transcriptome Analysis Group).

The particle bombardment was performed by using the protocol of Marzin et al. (2008). Bombarded leaf segments were incubated in closed Petri dishes in a climatized room (24 °C constant temperature, indirect light). At 24 h post-bombardment, green fluorescent protein (GFP)-expressing epidermal cells were counted by using an Olympus SZX12 stereo microscope (Olympus Europa GmbH, Hamburg, Germany).
After counting the GFP expressing cells the following stress treatments were applied.

For the *dehydration stress treatment* 50 % of the leaf segments were placed onto filter paper (Whatman 3MM CHR, GE Healthcare, Maidstone, Kent, UK) and air-dried to a fresh weight of 60 % relative to the initial fresh weight measured before the dehydration treatment. The dehydration was carried out in a laminar air flow box (Gelaire, Italy) at 22 °C and lasted 60±30 min. During this period the control leaf segments remained on water–phytoagar. After the dehydration-stressed leaves had reached the desired relative fresh weight (RFW), the filter paper was moisturized with 0.25 mL of H_2O and the Petri dishes were closed with Parafilm® (Pechiney, Chicago, IL, USA). The dehydration-stressed and control leaf segments were incubated for 96 h. At the end of the stress treatment, DsRed-expressing epidermal cells were counted in control and dehydration-stressed leaves under the microscope.

For the *salt stress treatments*, 50 % of the leaf segments were placed onto 0.5 % (w/v) water–phytoagar (Duchefa Biochemie, Harleem, The Netherlands) containing 175 mM NaCl and 10 mg L^{-1} benzimidazole (Sigma, St. Louis, MO, USA).

For the *methylglyoxal (MG) induced direct carbonyl stress treatments*, 50 % of the leaf segments were reposed onto 0.5 % (w/v) water–phytoagar (Duchefa Biochemie, Harleem, The Netherlands) containing 12.5 mM methylglyoxal (MG) (Sigma, St. Louis, MO, USA) and 10 mg L^{-1} benzimidazole (Sigma, St. Louis, MO, USA).

For each stress treatment the other 50 % of leaf segments were incubated on water-phytoagar serving as non-stressed controls.

Analysis of transgenic seed size by pixel analyis

80-80 seeds of the T2 plants were measured individually with Ohaus Model EP114C analytical balance by 0.1 mg accuracy. Size analysis of the same seeds was performed using the Seed Size Analysis Program (SSAP) v. 0.95 of the Plant Complex Stress Diagnostic System developed by László Sass at the BRC.

Quantitation of GFP and DsRed expressing cells by fluorescence microscopy

For GFP and DsRed detection, single-cell fluorescence was examined 24 h (GFP) or 120 h (DsRed) after biolistic bombardment using a fluorescence Olympus SZX12 stereo microscope (Olympus Europa GmbH, Hamburg, Germany) with 0.5x and 1x objectives. GFP and DsRed images were captured with green and red filter sets, respectively. Photos were captured using Olympus Camedia C7070 digital camera (Olympus Europa

GmbH, Hamburg, Germany) using DScaler software (version 4.1.15, www.dscaler.org).

Measurement of chlorophyll and carotenoid content

Approximately 300 mg leaf samples were homogenized in ice-cold 80 % (v/v %) aceton (10 mL/samples). The tubes were centrifuged at 2500 rpm for 10 min at 4 °C (Heraeus Labofuge 400R, Thermo Scientific, Walthman, USA). The supernatants were collected after centrifugation; pigment composition was measured by a Hitachi U-2900 spectrophotometer (Hitachi Ltd., Tokyo, Japan) according to Yang et al. (1998).

Statistical analysis

The statistical significance of the results was determined using the Microsoft Excel 2003 software (Microsoft Inc., Redmond, WA, USA) Student's T-test ($P \leq 0.05$).

RESULTS

Expression analysis of barley candidate genes for drought tolerance

We investigated the role of two barley genes (*HvARH1* and *HvSRG6*) in the adaptation to drought by gene expression analysis in barley genotypes of differentially drought tolerant haplotypes.

In the case of the *HvARH1* gene, the investigated barley haplotypes could be clustered into three main groups: (HvARH1-Z1, HvARH1-Z4 és a HvARH1-Z6); the decisive feature was a 6 bp long deletion in their promoter sequence in the Z4 and Z6 haplotypes, and a 9 bp long insertion in the Z1 haplotype's promoter. The effect of the insertion-deletion mutations were analysed in 3 genotypes in each haplotypes, respectively. We experienced massive gene induction in all the cases during ABA treatment. The strongest and most conform induction (20-60-fold increase) was observed in the HvARH1-Z1 haplotype. The cultivars belonging to the Z4 and Z6 haplotypes showed lower, 10-fold expression increase. An extremely high value was measured in the case of a *Compana* genotype belonging to the Z4 haplotype group demonstrated the importance of individual genomic characteristics and the necessity of the statistical analysis of data derived from multiple genotypes.

In the case of the ABA induction of *HvSRG6* gene, 5 barley genotypes containing the full length promoter and 3 genotypes (Otis, Chilga, Diamond) containing a 197 bp long deletion in the promoter were tested. Despite the reported abscisic acid inducibility of this gene, we observed only a very moderate response in *HvSRG6* expression that ranged from a maximal 2.7-fold induction to a 0.7-fold repression.

Production of MsALR expressing barley lines

To examine the in vivo stress-protective function of the *Medicago sativa* aldose reductase protein (*MsALR*) produced in different subcellular compartments of barley, transgenic plants producing this enzyme – targeted to the cytosol or the chloroplast – were generated. In paralell with the preparation of the expression cassettes, we succesfully adapted the *Agrobacterium*-mediated transformational method of barley embryos to our laboratory conditions. We proved the successful transfer of the target gene and the antibiotic resistance marker gene (*hpt*) using PCR technique, and with Western blot we verified both the level and the location (cytosolic or chloroplast-targeted) of the transgene's expression. We created 24 independent lines (14.3 % transformational efficiency) with cytosolic and 5 independent lines (4.4 % transformational efficiency) with chloroplast-targeted MsALR overexpressing barley lines. Firstly, transgenic plants were

tested for the presence of the integrated *MsALR* and *HPT* (hygromycin phosphotransferase) genes by PCR reactions. Due to the stringent selection protocol applied after Agrobacterium-mediated transformation, almost all regenerated barley transformants were positive in this assay. The results have indicated that the transgenic plants could accumulate similar levels of chloroplast targeted MsALR enzyme compared to cytosolic transformants. The transit peptide of the barley Rubisco small subunit ensured the efficient targeting of the fusion protein to the chloroplast, the level of protein accumulation was close to the transformants that accumulated the enzyme in the cytosol.

Selecting the candidate lines for the further experiments the transgene copy number was also determined in the case of the cytosol-targeted lines. Despite the characteristically low copy number of the *Agrobacterium*-mediated transformation, we experienced not only high expression, but also high copy number of the candidate gene (14.9 ± 2, and 11.2 ± 0.84, real-time qPCR technique).

Evaluation of the stress-protective effect of *MsALR* accumulation in transient assay system

As an informative cellular stress tolerance parameter, DsRed/GFP ratios were calculated in the fluorescent transient assay system in all of the independent experiments. In all of stress treated transgenic leaves, MsALR expressing barley lines (p6d35S-MsALR (cytosolic) és p6d35S-cpMsALR (chloroplast targeted)) showed significantly higher DsRed/GFP ratio than the treated GP plants at the end of the experiments.

The chlorophyll and carotenoid contents are the major stress sensitive components in the plant cell. The results of the calculations of DsRed/GFP fluorescence ratios were supported by the measurement of the chlorophyll and carotenoid contents of non-stressed and stress-treated samples. We found significant differences in chlorophyll carotenoid contents in the case of dehydration and salt stresses, the MsALR overexpressing barley lines retained more pigments compared to the GP; in transgenic barley significantly higher chlorophyll content was observed even under non-stressed conditions compared to the GP value. The MG-treated transgenic lines performed smaller decrease of the chlorophyll content than the treated GP samples. The carotenoid contents of dehydration stressed GP and MsALR transgenic lines were similar with a slightly higher value for the transgenic line. In salt and carbonyl stresses, there were undetectable changes in the carotenoid content between the values of GP and transgenic lines, however, the carotenoid content increased in both tested lines compared to the leaves placed under control conditions.

Our results demonstrated that compared to GP plants transgenic barley plants accumulating the MsALR protein either in the cytoplasm or in the chloroplast had significantly higher tolerance to diverse stresses. The observed stress protective effect of the produced MsALR protein was on a similar level in both the cytoplasmic and in the plastidic accumulation.

Barley transformations for *GW2* gene silencing

The next part of my thesis focuses to the experiments oriented to the silencing of the barley *GW2* gene variants, which play role in the grain size determination. First we identified the two barley homologues of the rice *GW2*, then we decreased the amount of the transcripts in transgenic plants using antisense gene silencing technology. Database searches have revealed that the barley genome encodes at least two *GW2* homologues. Our results have demonstrated that GW2-like proteins can be classified into three groups (dicot, *monocot I* and *monocot II*) in mono- and dicot species. Dicots contain two GW2 proteins with high level of homology, while monocots contain at least two GW2 proteins that shares considerable homology only in the RING-domain. We must note that until now no information is available on the function of the second group of monocot GW2 proteins, all published results were based on the characterization of *GW2* genes coding for the proteins belong to the *monocot I* group. In the case of the *ASHvGW2.1* construct we regenerated 14 plants that belonged to 4 independent transgenic lines, and in the case of the *ASHvGW2.2* construct 8 plants were regenerated from 2 independent lines. In these candidate lines we verified the transgenes' (target and marker genes) prescence by PCR, and measured the decreased expression level of target genes by real-time qPCR. In the T2 generation we performed a detailed charachterization and phenotypic analysis of the most important spike parameters (number of the main and secondary shoots, seed number, thousans kernel weight, length and width of the seeds). Although the silencing of the closer homologue to the rice *GW2* (*ASHvGW2.1* construct) did not cause strong phenotypic changes in the vegetative phase, the plants produced spikes earlier and the ripening of the seeds was significantly slower, leading to better grain-filling and larger seeds and higher yield. However, the silencing of the other homologue (*ASHvGW2.2* construct) appeared to provoke remarkable changes already in the vegetative growth phase of the barley plants. The plants were shorter, but the leaves were significantly larger, and the dry weight of the leaves increased with 80% compared to the wild type. The spike production was late; the spikes were covered by the leaves in full ripening. We observed differences between the number of the main and secondary shoots, and in the seed size in the two different transformant groups. In the other homologue we counted more secondary shoots that means the tilling was more vigorous. The

thousand seed weight was significantly higher in both groups, which could be explained with the increased width of the seeds, since we could measure only small differences in the seed length compaired them to the wild type ones.

CONCLUSIONS AND RECOMMANDATIONS

Expression analysis of barley candidate genes for drought tolerance

Published results have suggested that in certain crop plants the gene expressions of the aldo-keto reductases are different in stress tolerant and sensitive cultivars (Karuna Sree et al. 2000). In our experiments we tried to find a similar pattern, and also tried to determine the genomic background of variable tolerance levels of the cultivars. In connection, we investigated the role of two barley genes (*HvARH1* and *HvSRG6*) in the adaptation to drought by gene expression analysis in barley genotypes of differentially tolerant haplotypes.

Based on the literature data the expressional responses of both candidate genes were investigated during ABA treatment. The results of the published studies showed that the expression of the *HvARH1* gene is influenced by developmental and hormonal condition: the abscisic acid increases and the gibberellic acid obstruct the expression level of this gene (Bartels et al. 1991, Roncarati 1995). An extremely high value was measured in the case of a *Compana* genotype belonging to the Z4 haplotype group demonstrated the importance of individual genomic characteristics and the necessity of the statistical analysis of data derived from multiple samples.

In the case of the ABA induction of *HvSRG6* gene, 5 barley genotypes containing the full length promoter and 3 genotypes (Otis, Chilga, Diamond) containing a 197 bp long deletion in the promoter were tested. We observed only a very moderate response to ABA treatment in *HvSRG6* expression. This can be explained by the fact that PlantCARE analysis of the promoter showed that none of the sequence elements responsible for ABA or drought responsive gene expression could be localized to the deleted region.

Generally, we can conclude that the *SRG6* gene expression is high in genotypes where many other physiological characters (transpiration, the photochemical efficiency of PSII, membrane stability) are also less sensitive to physiological changes caused by drought stress (Rapacz et al. 2010). This candidate gene forms part of the complex mechanisms against drought stress (Xu et al. 2010; Tong et al. 2007). The analysis of other physiological parameters can lead to form more comprehensive opinion about the genotype-dependent gene expression pattern.

Production of MsALR expressing barley lines

In the case of cytosolic transgenic line (L3), the transgene copy number was determined by the most reliable method (qRT-PCR, Gadaleta et al. 2011). Despite of the experience that the *Agrobacterium*-mediated transformation results in low copy integration in general (Bartlett et al.

2008), we experienced not only high expression, but also high copy number of the candidate gene (14,9 ± 2, and 11,2 ± 0,84, real-time qPCR technique). The reverse orientation of transgene copies may lead to genetic instability, recombination events or gene silencing (Wolffe et al., 1997, Travella et al. 2005). Hernandez-Garcia et al (2010) reported a positive relationship between the GFP transgene copy number and the amount of the produced GFP protein during examination of new soybean promoters. It is therefore not unique that high levels of the protein could be detected in the case of transgenic lines with multiple MsALR copies.

In our case, the high transgene copy number leaded to high gene expression level. In the scope of our previous work (Oberschall et al., 2000) a specific polyclonal antibody was raised in rabbit against the recombinant MsALR protein. This antibody could recognize the produced MsALR in transgenic barley plants and showed negligable background on protein extracts of untransformed Golden Promise plants, moreover, the detected levels of the MsALR protein showed good corelation with the gene expression data, therefore we routinely use the Western blot analysis to characterize our transgenic plants.

The high transformation efficiency demonstrated another benefit of the Agrobacterium mediated transformation; that this method often results in stable transgene expression. The comparison of gene expression ratios caused by biolistic or *Agrobacterium tumefaciens* transformation method reviewed by Travella et al. (2005). Their results showed that 25 % of the transgenic lines produced by biolistic method resulted in transgene expression, while this ratio was 71 % of the lines generated by Agrobacterium- mediated transformation.

Evaluation of the stress-protective effect of *MsALR* accumulation in transient assay system

The stress tolerance experiments were carried out according to the literature's protocol using detached leaves from transformed barley plants. We improved the test system that is based on the transient expression of fluorescent proteins (GFP and DsRed). The most important advantage of this system is that it can be adapted easily for other stresses, and, due to its sensitivity, it can give us information about the scale of the stress tolerance in difficult situations where other methods such us photosynthetic mesurements cannot provide such data.

We characterized the stress protective effect of cytosolic and chloroplast-targeted *Medicago sativa* aldose reductase (*MsALR*) in transgenic barley plants. The selected transgenic lines showed higher DsRed/GFP ratio compared to the wild type, and they exhibited higher resistance to the

stresses, as well. The overproduction of MsALR enzyme can provide a defense against a wide range of abiotic stresses, because this can reduce the toxic level of the reactive aldehydes generated by dehydration, salt and carbonyl stresses (Oberschall et al., 2000). Recently we have demonstrated the stress protective action of the overproduction of MsALR enzyme in the cytoplasm of transgenic wheat plants (Fehér-Juhász et al., 2014).

We investigated the changes of the photosynthetic activities of detached leaves during the dehydration stress treatments. The Fv/Fm and [Y (II)] parameters were determined by fluorescence kinetics measurements (data not shown) in three different timepoints of the expreiments: at the beginning of the experiments, then directly after the bombardment, and at the end of the stress treatments. Although the results showed similar data than the GFP / DsRed ratios and the chlorophyll content, a similar result was provided (the transgenic lines showed higher parameters at the end of the stresses compared to the GP), but these differences did not proved to be statistically significant compared to the wild-type Golden Promise leaves. It should be mentioned, however, that the transgenic barley accumulating the MsALR enzyme in the cytoplasm can successfully decrease the effects of reactive aldehydes. However, this may only indirectly protect the photosynthetic apparatus; this is why we could get a significant difference during the test, despite the photosynthetic parameters showed no significant difference (data not shown).

The results of these different stress experiments suggested that the decrease of the normalized number of DsRed fluorescent cells is a valuable parameter that can mirror the cellular stress caused by dehydration or salt and chemical (carbonyl) stress. These results also indicated that the transient assay system is sensitive enough to show the cellular level stress tolerance of stable transgenic lines in a wide variety of stresses. The tetramer formation (and thus the fluorescence) of DsRed protein depends on cytosolic conditions that respond sensibly to intracellular stress that may be translated to other physiological responses in a delayed manner. The method can also be applied to non-photosynthetic tissue–on roots, that can be particularly important for drought response evaluation or on cell suspension–moreover it enables the evaluation of the cellular consequences of stresses in transgenic barley as well as in natural barley cultivars.

Since chlorophyll and carotenoids are the major components in the plant cell that are sensitive to stress, Parida and Das (2005) suggested that a decrease in the chlorophyll content in response to salt stress is a general phenomenon which led to the failure of chlorophyll synthesis and induced chlorosis in plants, similarly to drought stress (Kirnak et al. 2001). Carotenoids also play a critical role in the assembly of the light-harvesting complex and according to their antioxidant activity within the chloroplasts; these pigments play a role in scavenging singlet oxygen as well as

preventing lipid peroxidation. In this present study, the chlorophyll and carotenoid content of the control and stressed detached leaves was determined in order to support the stress tolerance estimations obtained by the fluorescence protein-based method. All kinds of stress treatments lead equally to the reduction of chlorophyll content. In the case of dehydration or salt stresses we found, that the chlorophyll levels of the stress treated transgenic leaves were much higher than in the GP plants, which suggests, that the overproduction of MsALR lead to increased stress tolerance against the applied stresses, partially through the preservation of the photosynthetic apparatus system. In the case of the methylglyoxal stress, the treated transgenic line demonstrated only 5 % higher photosynthetic pigment content compared to the stress treated GP samples. This may indicate that the protective effect of the detoxifying enzyme is not only manifested on the components of the photosynthetic apparatus, but the direct intracellular reduction of MG can significantly decrease the possibility of the damage of cytosolic proteins as well. Looking at the carotenoid content in both transgenic and non-transgenic plants, an increase was found after the dehydration treatment, but the salt and MG stress experiments did not follow this trend; the carotenoid content increased or did not change in all tested lines compared to the non-stressed leaves. This phenomenon can be explained with the plants' ability to synthetize enough carotenoids that can be utilized for the protection of the proteins of the photochemical system (via non-photochemical quenching of chlorophyll fluorescence).
Our results demonstrated that compared to GP plants, transgenic barley plants accumulating the MsALR protein either in the cytoplasm or in the chloroplast had significantly higher tolerance to diverse stresses. The observed stress protective effect of the produced MsALR protein was on a similar level in both the cytoplasmic and in the plastidic accumulation. This may originate from the fact that the production of the enzyme's natural substrates, the reactive carbonyl compounds can occur in both intracellular locations. Such compounds can be reduced in the chloroplast directly by PSI, but the importance of their enzymatic elimination by AKRs increases under stress or low light conditions.

Barley transformations for *GW2* gene silencing

The next part of my thesis focuses to the experiments oriented to the silencing of the barley *GW2* gene variants, which play role in the grain size determination.

The silencing of the ulterior *GW2* homologue (*ASHvGW2.2* construct) appeared to provoke remarkable changes already in the vegetative growth phase of the barley plants. The plants were shorter, but the leaves were significantly larger, and the dry weight of the leaves increased

compared to the wild type. The spike production was late; the spikes were covered by the leaves in full ripening. We observed differences in the number of the main and secondary shoots and in the seed size in the two different transformant groups. In the other homologue we counted more secondary shoots, which means the tilling was more vigorous. The thousand seed weight was significantly higher in both groups, which could be explained with the increased width of the seeds, since we could measure only small differences in the seed length compaired them to the wild type ones. This finding is well-harmonized with the data in the literature (Song et al. 2007).

These results allow us to conclude that these ubiquitin ligases can play pivotal role during the switch from vegetative to generative phase. The analysis of the applicability of this gene as a seed size determining marker is a future aim, using indipendent triticale DH lines possessing different sized seeds. Also, it could be interesting to investigate this gene's expression in dicot plants due to its possible effect on biomass production.

New scientific results

1. Transcriptional responses of two candidate genes (*HvARH1* and *SRG6*) were investigated to ABA treatment. In our experiments the induction of the *HvARH1* gene expression by ABA has shown considerable differences in the three haplotypes tested. The gene expression was the most pronounced in the case of genotypes belonging to the HvARH1Z1 haplotype group. In the case of the *HvSRG6* gene, despite the reported ABA inducibility of this gene, we observed only a very moderate response in *HvSRG6* expression. These minor changes in transcript levels did not correlate with a very pronounced change in the gene's promoter.

2. Barley transformation experiments were performed using a reactive aldehyde deactivating *ALR* gene isolated from *Medicago sativa*. In these experiments we produced transformed lines targeting this protein into the cytosol and the chloroplast, respectively. In paralell with the preparation of the expression cassettes, we succesfully adapted the *Agrobacterium*-mediated transformational method of barley embryos to our laboratory conditions. We created 24 independent lines (14.3 % transformational efficiency) with cytosolic and 5 independent lines (4.4 % transformational efficiency) with chloroplast-targeted MsALR overexpressing barley lines. Selecting the candidate lines for the further experiments the transgene integration and expression, transgene copy number was also determined in the case of the transgenic lines.

3. The modified version of fluorescent protein based transient assay system can be utilized for the evaluation of stress tolerance of MsALR producing stable barley transformants in their early vegetative developmental stage. The transgenic barley plants constitutively express the alfalfa *MsALR* gene and accumulates the produced protein in the cytosol or in the chloroplast. The results demonstrated that the dual fluorescent protein-based method can be successfully applied in a variety of stress conditions (dehydration, salt and carbonyl stresses) and the procedure is suitable for testing the cellular stress tolerance of the stable transgenic genotypes in their early developmental stages and/or allows the physiological comparison of different stress tolerant varieties and transgenic lines. Our results also demonstrated that the increased reactive carbonyl detoxification capacity in the chloroplasts, provided by the targeted MsALR enzyme, can improve the stress tolerance of transgenic plants similarly to its cytoplasmic accumulation.

4. We identified the two barley homologues of the rice *GW2*, then we decreased the amount of the transcript in transgenic plants using antisense

gene silencing technology. In the case of the *ASHvGW2.1* construct we regenerated 14 plants that belonged to 4 independent transgenic lines, and in the case of the *ASHvGW2.2* construct 8 plants were regenerated from 2 independent lines. In these candidate lines we verified the transgenes' (target and marker genes) prescence by PCR, and measured the decreased expression level of target genes by real-time qPCR. We observed increased plant height in both transformant groups. The thousand seed weight was also significantly higher in both groups, which could be explained with the increased width of the seeds, since we could measure only small differences in the seed length compaired them to the wild type ones.

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