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ARTICLE

Transient and transgenic approaches for functional testing of candidate genes in barley

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ABSTRACT Barley represents one of the major crops grown worldwide. More than ten years have passed since the first stable Agrobacterium-mediated barley transformation was reported. Since then, several progresses have been made thanks to a number of technical improvements. As a result of our established barley transformation protocol, five different candidate genes were used for barley transformation. The transgenic lines were analyzed at biochemical and physiological levels. The aim of our work was to develop an effective and reproducible system for the analysis of transgenic lines (transient assay system in microprojectile bombarded barley leaf) which can be applied for testing the cellular stress response or cellular stress tolerance of the stable transgenic genotypes compared to the wild type barley. According to the results of the candidate gene testing in the transgenic lines, the investigated gene significantly increased the tolerance to the applied (drought) stress. Further experiments showed that these significant differences in the transient GFP/DsRed expression may correlate to the stress tolerance of the transgenic line.
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KEY WORDS

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candidate genes
drought stress
GFP/DsRed transient assay

Barley (*Hordeum vulgare* L.) is one of the most important crops in the world. Recently it has been used as a genetic model system because of its true diploidy along with the similarity of its genome to that of other small-grain cereals.

The first report on stable barley transformation via Agrobacterium was published in 1997 by Tingay et al. using the model cultivar Golden Promise. The method that we established to our laboratory conditions was developed for this variety and it's based upon protocols by Trifonova et al. (2001) and Kumlehn *et al* (IPK, Gatersleben, unpublished). In the cloning procedures, the constructs for plant transformation were ligated into pUbi-AB vector for ubiquitin promoter-driven expression. The binary vector for Agrobacterium (LBA4404 strain) transformation of barley plants was the p6d35S vector (Kumlehn et al. unpublished) (Fig. 1A). The established barley transformation technology is important for the functional characterization of candidate genes and the produced transgenic lines are subject for further studies.

Candidate genes used for barley transformations.

Candidate genes were selected based on their identified and putative role in the cell cycle regulation, grain size determination and in molecular drought response. One of these candidate genes, the *MsCDKB2;1* a *Medicago sativa* cyclin dependent kinase is regulator of the cell division cycle and plays a central role in regulation of G2/M phase transition. Previously, it was demonstrated that the overproduction of the enzyme resulted in significant changes in agronomically important characteristics in transgenic rice (Lendvai, PhD thesis). Other candidate genes, like OsPP2A B" regulatory subunit, *OsRBRI2* and *OsRBRI5* are interactors of rice retinoblastoma-related protein, OsRBR1. The *GW2* was selected on the basis of its influence on the grain size determination. Loss of *GW2* function increases grain weight, width and yield (Song et al. 2007). Antisense approach resulted increased grain size, even with constitutive expression of the gene fragment in transgenic rice. We have identified and cloned the homologous gene from barley, a specific fragment of this was used for the generation of *HvGW2* antisense plants. All these transgenic barley lines will be analyzed for changes in growth and yield parameters.

A thoroughly analyzed candidate gene belongs to the group of oxidative stress-defense genes. First transformation was made using the alfalfa aldo-keto reductase, *MsALR*, isolated and analyzed previously in our laboratory (Oberschall et al. 2000). The members of the aldo-keto reductase superfamily can detoxify lipid peroxide degradation products and reduce the cytotoxic methylglyoxal to acetol. In addition to their role in the detoxification processes, these enzymes may produce compatible solutes. Over-production of such enzymes can increase the efficiency of mechanisms for reducing cellular damage by eliminating toxic reactive aldehyde products from the cells after oxidative stress and helps the recovery of the plants (Oberschall et al. 2000).

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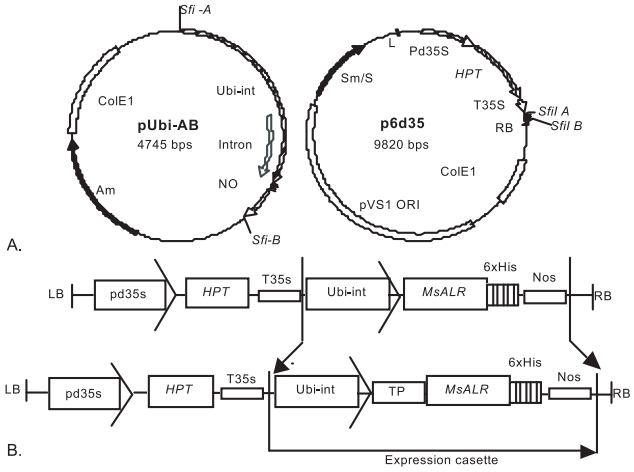

Figure 1. Barley transformation vectors (A) and constructs for cytosolic and chloroplast targeting of the MsALR protein (B): schematic maps of the T-DNA region of the plant expression vector. pUbi-AB: intermediate vector, p6d35s: plant expression vector, pd35s: double 35s promoter, *HPT*: hygromycin phosphotransferase gene, T35S: CaMV35S terminator, Ubi-int: ubiquitin promoter/intron, TP: Barley Rubisco Small Subunit Transit Peptide, *MsALR*: Medicago sativa aldo-keto reductase gene, 6xHis: 6xHis tag, *Nos*: 3UTR of nopaline synthase gene, Col1: Origin of replication of ColE1, pVS1 ORI: Origin of replication of pVS1. (The vectors pUbi-AB and p6d35s were provided by the IPK Gatersleben, Plant Reproductive Biology Group).

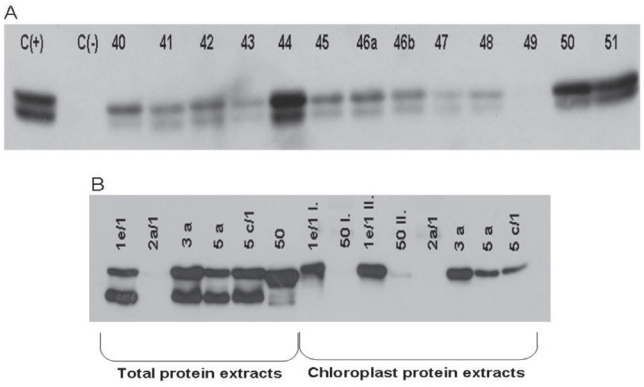

Figure 2. MsALR protein is accumulated to high level in different cellular compartments of transgenic plants. A: Western analysis of transgenic barley line accumulating MsALR protein in the cytosol. + : Positive control sample (transgenic tobacco), - : Negative control sample (wild type barley, Hordeum vulgare conv. Golden Promise), 40-51: total protein extract from transgenic barley lines. B: Comparative Western analysis of the total and chloroplast protein extracts of transgenic barley lines accumulating the MsALR protein either in the cytosol or in the chloroplast. 50: transgenic barley line which accumulates MsALR protein in the cytosol;. 1e/1, 3a, 5a, 5c/1 : transgenic barley lines which accumulates MsALR protein in the chloroplast, 2a/1 : non-transformed line.

The main source of reactive oxygen species (ROS) in plants is the chloroplast, so the detoxification enzymes should be found in high concentration in this subcellular compartment. In order to accumulate the cytosolic MsALR enzyme in the chloroplast, we constructed a vector for chloroplast targeting of the protective enzyme using the transit peptide encoding region of the barley *Rubisco SSU* gene (Fig. 1B). Thus we can investigate whether the accumulation of the protective enzyme in different subcellular compartments could influence the level of tolerance to abiotic stresses.

In cellular level, the stress response can be initiated by several environmental factors that cause damage to biological macromolecules including lipids, proteins (Kültz 2005). The cellular stress response associated with a series of transcriptomic and metabolic events leading to the accumulation of protective proteins that may counteract the stress-induced damage, and increase cellular tolerance to such factors. Our aim was to establish an efficient barley transformation protocol and to improve an effective and easily reproducible analytic system which can be applied in various stress and which is suitable for testing the cellular stress tolerance of the stable transgenic genotypes.

Materials and Methods

Plant material

Seeds of *Hordeum vulgare* conv. Golden Promise and the transgenic barley line carrying the *MsALR* gene derived from *Medicago sativa* (Oberschall et al. 2000) were grown in greenhouse at 18/16°C day/night temperature with a 12-h photoperiod and ambient relative humidity. For the Agrobacterium-mediated barley transformation, the protocol of Hensel et al. (2004) was followed.

Protein extraction and Western blot analysis

For total protein extraction, two leaf segments from each plant (50 mg fresh weight) were pulverized in liquid nitrogen, and they were homogenized in 200 µl of isolation buffer (Oberschall et al. 2000). For chloroplast protein extraction, approximately 50 mg leaf segments were cut and the procedure was performed according to protocol of Kley et al . (2010). Same amount of protein (10 µg) from each sample was loaded on a 12% SDS poliacrylamyde gel, and then blotted onto Immobilon-P PVDF membrane (Millipore). For the immunological detection of the expressed protein the α-MsALR polyclonal antibody and the peroxidase (POD) conjugated anti-rabbit IgG antiserum were used as described in Oberschall et al. (2000).

Microprojectile bombardment and stress treatments

For the transient assay system, the microprojetile bombardment and the dehydration stress treatment protocol of Marzin et al. (2008) were followed. The DsRed/GFP ratio was finally calculated in the control and stressed leaf segments (Fig. 3).

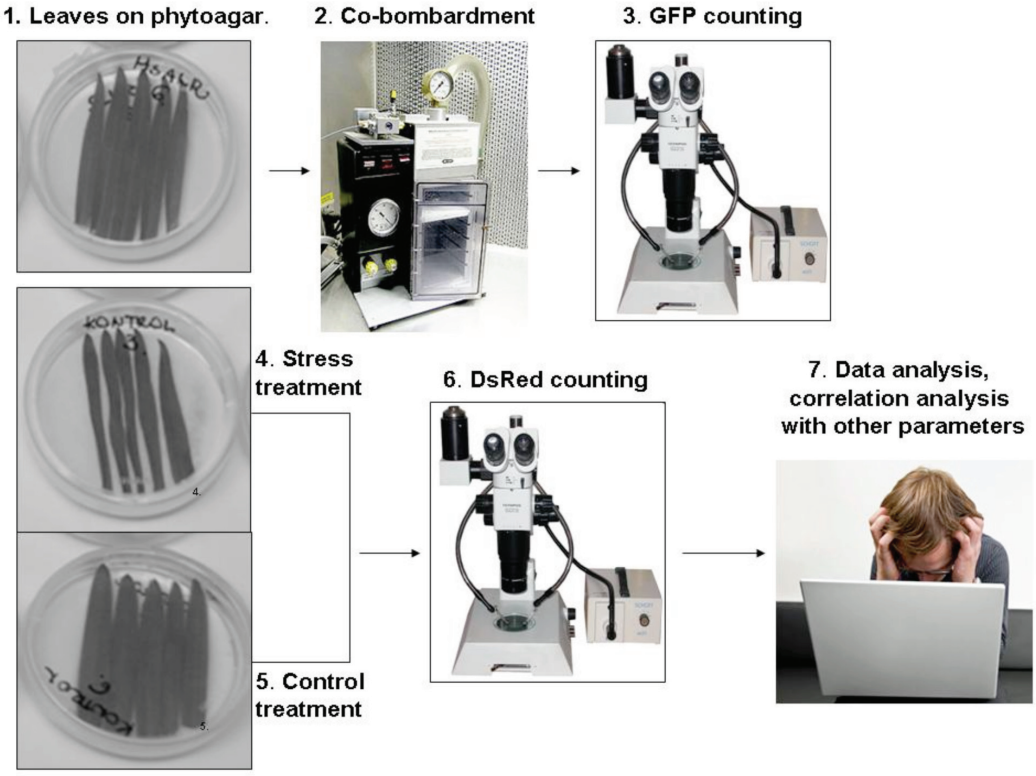

Figure 3. Transient assay system in microprojeticle bombardment barley leaf. 1. 7-d-old cut leaf on water-phytoagar, 2. Co-bombardment with the GFP and DsRed expression constructs, 3. At 24 h post-bombardment, microscopical counting of the green fluorescent protein (GFP)-expressing epidermal cells, 4. Stress treatments (dehydration) and 5. Control leaf segments remained on water–phytoagar and maintenance the stress for 4 days, 6. Microscopical counting of the red fluorescent protein (DsRed)-expressing epidermal cells, 7. Calculation of the GFP/DsRed ratio in non-stressed fully turgescent and stress treated samples, analysis of other biochemical and/or physiological data.

Chlorophyll isolation

Five primary leaf segments were extracted with 80% acetone, ground and centrifuged then chlorophyll (chlorophyll *a+b*) and carotenoid contents were calculated based on absorbances at 664.6, 646.6 and 440.5 nm according to Yang et al. (1998).

Results and Discussion

The barley transformation method was successfully established in our laboratory. As Table 1 shows more than 10% of the co-cultivated embryos resulted transgenic plants.

The *MsALR* transformed plants were analysed by Western blot analysis (Fig. 2). The results have demonstrated that the majority of the transformed line accumulates the transgene-encoded protein and the protein level in some of the transgenic lines reaches that of the positive control transgenic tobacco plants.

Dehydration or drought stress generates complex molecular and physiological changes in plant cells and the oxidative destruction of the lipid membranes can amplify cellular toxicity by the formation of lipid hydroperoxides and toxic aldehyde degradation products. In a transient assay study, the biochemical features of the red fluorescent protein (DsRed) were applied to investigate drought stress tolerance in bombarded epidermal cells of barley. DsRed is known to be sensitive to denaturing conditions and to require several days for maturation into the fluorescent homo-tetramer complex. The amount of mature, fluorescent DsRed will be reduced according to the level of severely stressed cells.

For the first experiments we used wild type barley plants to adopt the microprojectile bombardment system. When the dispersion of the gold particles was sufficient on the surfaces of leaves, one of our transgenic plants (*MsALR*_50

Table 1. Transformation efficiency calculation of the MsALR transformed plants (IE: isolated embryo).

Construct: p6d35s-MsALR	
Number of IEs	180
Number of calli formed from the IEs	168
Number of verified transgenic plants	25
Transformation efficiency	13.88%

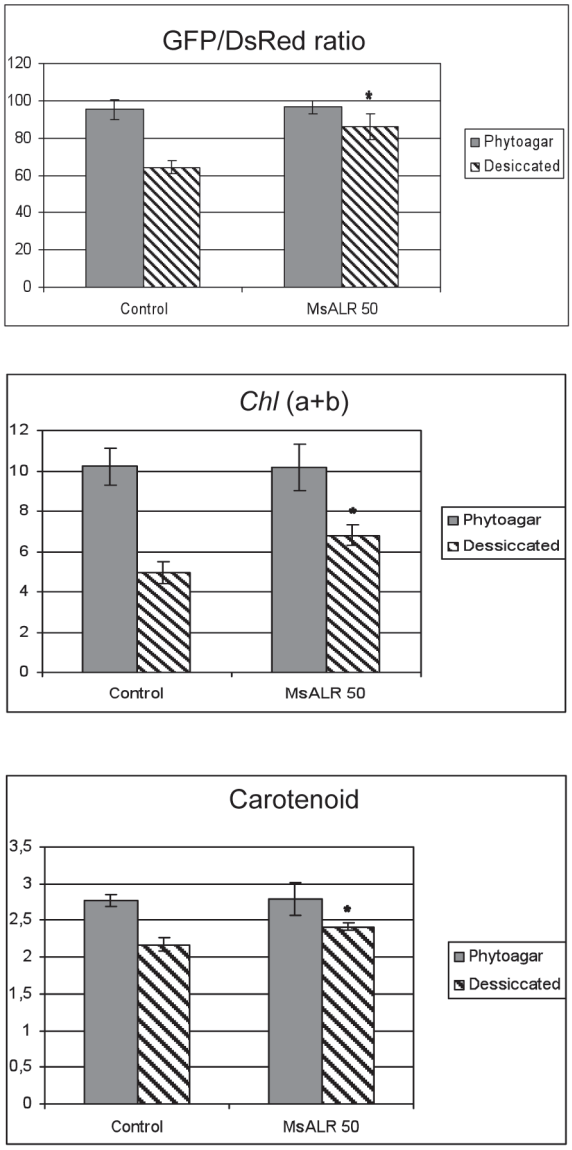

Figure 4. Result of the GFP and DsRed ratio counting, chlorophyll and carotenoid content measurements in fully turgescent and in dehydration stressed leaf segments. Significant differences (at P\0.05) from control plants are labelled with asterisk.

line, Figure 2) which have got high transgene expression was choosen for test in this system. After the bombardment, GFP was allowed to accumulate in non-stressed conditions. 24 hours later the GFP expressing cells were counted. This was followed by the stress treatments 50% of the leaf segments were desiccated until their relative fresh weight has reached the 60 % of the initial weight. The second 50% of the leaf segments were kept on water-phytoagar, as fully turgescent, non-stressed control (Marzin et al. 2008). All of treated and non-treated leaf segments were incubated for 4 days. At the end of the desiccation stress period, the number of DsRed-fluorescing cells was counted and normalized by the GFP-expressing cell numbers that was detected in the same leaf segments before the beginning of the stress treatment. As a result, we obtained different GFP/DsRed ratios in all of independent experiments. We found only a moderate reduction of the number of DsRed fluorescent cells in the non- stressed, fully turgescent control and transgenic leaves, but the GFP/DsRed ratio of the desiccated transgenic *MsALR*_50 line was significantly higher than that we counted in the case of the desiccated control plants (Fig. 4). These results suggest 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 other stress treatments.

These results were supported by the analysis of chlorophyll and carotenoid content of the non-stressed and the stress-treated samples. We found significant differences between the aldo-keto reductase overexpressing plants and control plants (especially in the dehydration-stressed leaf) in the chlorophyll and carotenoid contents. In all of experiments, the transgenic lines performed better, compared to the control plants (Fig. 4).

Photosynthetic parameters of the detached leaves were measured directly before the bombardment, 24 hours after the bombardment, and finally, 120 hours after the bombardment. In some of the experiments the photochemical yield of PS II in the dark adapted state (Fv/Fm) and effective photochemical yield of PS II of leaves (Y (II) 55) represented similar differences that were shown by the DsRed/GFP fluorescence ratios and chlorophyll/carotenoid contents. However these data showed much higher level of error and inconsistency, therefore we suggest to use the latter parameters to estimate the level of cellular stress.

The extension of the DsRed/GFP-based transient system to salt and oxidative stress tolerance in transgenic barley lines might be a further option for the evaluation of the effect of trangenes on cellular stress tolerance.

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