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Stress tolerance of transgenic barley accumulating the alfalfa aldose reductase in the cytoplasm and the chloroplast

Bettina Nagy [a], Petra Majer [a], Róbert Mihály [b], János Pauk [b], Gábor V. Horváth [a, *]

[a] Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, 6726, Szeged, Hungary
[b] Cereal Research Non-Profit Company, Alsó kikötő sor 9, 6726, Szeged, Hungary

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ABSTRACT

Barley represents one of the major crops grown worldwide; its genetic transformation provides an important tool for the improvement of crop quality and tolerance to environmental stress factors. Biotic and abiotic stresses produce reactive oxygen species in the plant cells that can directly oxidize the cellular components including lipid membranes; resulting in lipid peroxidation and subsequently the accumulation of reactive carbonyl compounds. In order to protect barley plants from the effects of stress-produced reactive carbonyls, an *Agrobacterium*-mediated transformation was carried out using the *Medicago sativa* aldose reductase (*MsALR*) gene. In certain transgenic lines the produced MsALR enzyme was targeted to the chloroplasts to evaluate its protective effect in these organelles. The dual fluorescent protein-based method was used for the evaluation of tolerance of young seedlings to diverse stresses; our results demonstrated that this technique could be reliably applied for the detection of cellular stress in a variety of conditions. The chlorophyll and carotenoid content measurements also supported the results of the fluorescent protein-based method and the stress-protective effect of the MsALR enzyme. Targeting of MsALR into the chloroplast has also resulted in increased stress tolerance, similarly to the observed effect of the cytosolic MsALR accumulation. The results of the DsRed/GFP fluorescent protein-based method indicated that both the cytosol and chloroplast accumulation of MsALR can increase the abiotic stress tolerance of transgenic barley lines.

1. Introduction

Barley (*Hordeum vulgare* L.) is among the first domesticated grains and the most important crop plants in the world; its importance as an experimental model has been increasing because of its chromosome set that represents the base complement of species within the tribe *Triticeae*. Barley transformation—as a tool of modern plant breeding and biotechnology—could be useful for the improvement of crop quality. Results for the first, indirect barley transformation method were described by Tingay et al. (1997), who presented stable transgenic barley plants through co-cultivation of immature embryos with *Agrobacterium tumefaciens*. This method served as a basis for further improvements (Matthews et al., 2001, Trifonova et al., 2001); however, this kind of barley transformation method is still restricted to a few genotypes: the use of the Golden Promise (GP) cultivar susceptible for *Agrobacterium*-mediated transformation generates high reproducibility, transformation efficiency and transgene expression (Matthews et al., 2001, Hensel and Kumlehn, 2004).

The breeding strategy for barley is often addressed to enhanced stress tolerance, because environmental stresses are the principal causes of yield reduction; they generate considerable losses worldwide in agricultural production (Acquaah, 2006). The common feature of numerous biotic and abiotic stresses that they lead to the production of reactive oxygen species (ROS) (Apel and Hirt, 2004). Some of these compounds are highly toxic and they cause damage to DNA, proteins and lipid membranes; which results in further lipid peroxidation and the generation of degradation products (eg. reactive carbonyl compounds, RCOs). One possible route for the detoxification of RCOs is their reduction by aldo-keto reductase enzymes (AKR). The majority of the plant AKRs belongs

Abbreviations: AKR, aldo-keto reductase; cpMsALR, MsALR protein targeted to the chloroplast; DsRed, red fluorescent protein from *Discosoma* sp.; GFP, green fluorescent protein; HNE, 4-hydroxynon-2-enal; MG, methylglyoxal; MsALR, *Medicago sativa* aldose reductase; MV, methylviologen; OsAKR1, *Oryza sativa* aldo-keto reductase 1; *rbcS*, Rubisco small subunit gene; RCO, reactive carbonyl compound; ROS, reactive oxygen species.

* Corresponding author.
E-mail address: horvath.gabor@brc.mta.hu (G.V. Horváth).

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to the AKR4 family; mostly to the AKR4C subfamily. The first representative of this subfamily was cloned and isolated from barley (Bartels et al., 1991); one proven role of these enzymes is the reduction of reactive lipid peroxidation products such as methylglyoxal (MG) and 4-hydroxynon-2-enal (HNE) (Oberschall et al., 2000, Turóczy et al., 2011), which are generated in cells under oxidative stress. Removing HNE and MG from stressed cells has been proposed as a role for *Medicago sativa* ALR (MsALR) and this function was demonstrated in *Nicotiana tabacum* transformant plants (Oberschall et al., 2000). The results suggested that the increased synthesis of MsALR enzyme led to enhanced tolerance against drought and oxidative stress induced by heavy metals, methylviologen (MV), hydrogen-peroxide or ultraviolet-B irradiation (Oberschall et al., 2000, Hegedűs et al., 2004, Hideg et al., 2003). Transgenic tobacco plants overproducing a rice homologue, OsAKR1 showed increased heat tolerance and accumulated a lower level of MG and lipid peroxidation products due to the higher activity of this enzyme in leaves (Turóczy et al., 2011). MsALR overexpressing transgenic wheat plants were well characterized earlier throughout the whole vegetative and generative growth phase under permanent drought stress and control condition (Fehér-Juhász et al., 2014). These transgenic wheats consequently exhibited 1.5–4.3 times higher detoxification activity for the aldehyde substrate, their green biomass production was 12–41% higher than the non-transgenic plants' grown under water limitation. Thermal imaging of control and drought stressed plants provided a comprehensive data set demonstrating the improved physiological condition of the drought stressed transgenic wheat plants in the vegetative growth phase. In soil with reduced water capacity the transgenic genotypes showed higher seed weight per plant than the control non-transgenic one. These experimental findings formed a basis for the improvement of abiotic stress tolerance of transgenic barley plants by exploiting the aldo-keto reductase enzymatic activity against the stress-derived RCOs.

The above listed experimental results provided pursuading evidences on the protective effect of MsALR's overproduction under stress conditions, however the development of a new methology is necessary to estimate this protection at cellular level in young seedlings. Such tool can be advantageous in the early evaluation and selection of different stable transformant lines. The level of cellular stress caused by the accumulation of ROS and RCOs can be estimated using fluorescent proteins (FPs). A wide variety of such proteins is available to monitor developmental and spatial gene expression patterns in transgenic organisms. The biochemical properties of some FPs are suitable to signify the forthcoming alteration in the plant cell suffering from biotic and abiotic stresses. Specially, the red fluorescent protein from Discosoma sp. (DsRed) can mirror the strength of dehydration stress, because it requires several days of maturation of the native proteins into the fluorescent tetrameric complex in the reducing environment of the cytosol (Baird et al., 2000). During this maturation period the denaturing conditions and cell death can reduce the number of fluorescent DsRed containing cells. Another commonly used FP, the green fluorescent protein (GFP) has also special attributes; it could be easily expressed in almost all organisms and in most known cell types and (importantly from the viewpoint of the assay) its fluorescence is not influenced by cellular stress. Connected with these attributes of the above mentioned FPs, a transient, fluorescent protein-based stress response assay system was developed in barley for testing gene function under biotic stress caused by *Blumeria graminis* (Panstruga et al., 2003). However, in the literature, only limited studies are useable for testing the abiotic stress tolerance, the harmful effect of ROS for seedling or plantlets in cellular level. Short term stress treatments were often used for the evaluation of phenomics tools for testing dehydration stress-related genes of barley and determination of ABA level changes caused by dehydration as described earlier (Marzin et al., 2008,Grossi et al., 1995,Faltusová-Kadlecová et al., 2002). In Marzin et al. (2008); TIGS of four tested genes resulted in a significantly stronger decrease of normalized DsRed fluorescence in dehydration-stressed leaves, whereas they had no effect in fully turgescent control leaves. These genes encode barley drought-responsive factor *HvDRF1*, dehydrin 6, *HVA1*, and *HvHNX1*. The four targeted transcripts were also found to accumulate rapidly in severe dehydration-stressed barley leaf segments. RNAi constructs mediated transient induced gene silencing used for enhancing the susceptibility to dehydration stress in epidermal cells. Thus, this test system should be able to reveal is cellular survival, protection from protein denaturation and damage, plus enzymatic protein degradation. In *Arabidopsis*, a protoplast transient expression system was generated to elucidate the different signal transduction pathways underlying stress signaling (Sheen, 2001). However, a similar FP based system has not been used directly for testing abiotic or biotic stress tolerance of stable transgenic plants or different plant varieties in their early vegetative growth period.

In the present study, our aim was to investigate whether the fluorescent protein based transient assay system can be utilized for the evaluation of diverse stress tolerance of MsALR producing stable barley transformants in their early vegetative developmental stage. The transgenic barley plants constitutively expressing the alfalfa *MsALR* gene and accumulating 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.

2. Results and discussion

2.1. 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. Firstly, transgenic plants were tested for the presence of the integrated *MsALR* and *HPT* (hygromycin phosphotransferase) genes. Due to the stringent selection protocol applied after *Agrobacterium*-mediated transformation, almost all regenerated barley transformants were positive in this assay (Fig. 2a,b). The expression of the *MsALR* gene was examined by quantitative RT-PCR, the results have demonstrated considerable difference in the gene expression among the transgenic lines (Fig. 2c). 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 negligible background on protein extracts of untransformed Golden Promise plants (Fig. 2d,e). Moreover, the detected levels of the MsALR protein showed good correlation with the gene expression data (Fig. 2c,d); therefore we routinely use the Western blot analysis of our transgenic plants. The results have indicated that the transgenic plants could accumulate similar levels of chloroplast targeted MsALR enzyme compared to cytosolic transformants (Fig. 2d,e). Despite of the high

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