The role of rice aldo/keto reductases in the detoxification of reactive carbonyls and their use to create stress resistant transgenic plants

Summary of the PhD thesis

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Introduction

Stress conditions contribute to the disruption of balance between the oxidants and antioxidants, causing oxidative stress and cellular damage. Reactive compounds produced under such conditions (e.g. ROS) significantly increase the cytotoxic effect of environmental stress factors. The cellular damage caused by these radicals is mediated by their interactions with different cellular constituents, including lipids. Formation of lipid peroxides either chemically (lipid peroxidation) or enzymatically (glcolysis) is an integrated component of cellular damage during stress of the plants.

Aldose reductase, the first identified member of the aldo-keto reductase (AKR) superfamily, catalyses the conversion of glucose to sorbitol, the first step of the polyol pathway. Generally many AKRs are effective in the synthesis of osmolytes, thus having important role in the osmoregulation, which is an important process in plants for the acquisition of desiccation tolerance. Other than this function, members of the AKR family have also been shown to be effective in the detoxification of lipid peroxidation- and/or glycolysis-derived reactive carbonyls (e.g malondialdehyde (MDA), 4-hydoxy-nonenal, methylglyoxal (MG) etc.). Therefore, many AKR's have role in the protection of cells against the harmful effects of reactive carbonyls (e.g. protein modifications). The recently characterized AKR4C9 (*At2g37770*) from *Arabidopsis thaliana* supports the importance of the enzymatic action directed towards oxidative stress-linked reactive carbonyls, such as MDA. A good example showing the benefits of AKR enzymes are the transgenic tobacco plants overexpressing an alfalfa AKR protein (MsALR) with increased tolerance against a variety of oxidative stresses induced by methylviologen (MV), heavy metals, UV-B irradiation, osmotic and salt stress conditions and long periods of drought compared to the wild type plants.

Aims of study

The main aim of this work was the implementation of a transgenic approach (overexpression of a detoxifying enzyme) to confer multiple abiotic stress tolerance to model- and in the future to crop plants, through the action of AKRs, by enhancing the reactive carbonyl detoxification ability of the overexpressing plants. The results we obtained could be useful in the near future to create stress resistant crop genotypes (rice, wheat, barley); therefore they could have agronomical benefit. Here are the aims in more detail:

- Selection and identification of stress inducible rice *AKR* genes through different *in silico* methods (promoter analysis, homology searches, chromosomal localization) and to check their expression level in stressed rice cell suspensions.

- To monitor the induction of rice AKR proteins in treated (heat stress, H_2O_2, abscisic acid (ABA), benzyl alcohol) rice cell suspensions.

- To analyze the enzyme kinetic parameters of the protein coded by the most stress responsive gene, the OsAKR1 and to investigate both *in vitro* and *in vivo* the reactive carbonyl detoxification ability of this enzyme.

- To show the toxic effects of the protein carbonylation on the activity of the Calvin-Benson cycle's phosphoribulokinase (PRK), an important enzyme of the photosynthetic metabolism and thereby to emphasize the importance of our strategy; more effective reactive carbonyl detoxification, thus preventing protein modifications.

- To create stress resistant transgenic tobaccos through the heterologous overproduction of OsAKR1 and to evaluate the effects of overexpression on the abiotic stress tolerance of the transgenic lines.

- Ultimately the final purpose of this research for the near future is to obtain stress resistant crop genotypes (e.g. rice, wheat) through the overexpression of OsAKR1 using cis- and transgenic approaches.

Methods

1) *In silico* methods (genomic and proteomic databases, gene selection, homology tree, promoter analysis)

2) Molecular biology methods
- gene expression studies through Quantitative RT-PCR
- gene cloning
- genetic transformations (tobacco, *Escherichia coli*)
- recombinant protein expression, immunochemical methods
- enzyme kinetic measurements
- *in vitro* protein modification studies with reactive carbonyls

3) Physiological measurements
- spectrophotometric determination of MG
- TBARS test (measurement of the MDA levels)
- effective PSII quantum yield measurement with Mini PAM
- AKR activity measurement in total leaf protein extracts
- PRK activity measurements

4) Stress treatments
- on rice cell suspensions (ABA, H_2O_2, benzyl alcohol, NaCl)
- heat stress
- oxidative stress (MV)

Results

1) Selection of stress inducible rice *AKR* genes

Our work started with the search for highly homologous rice *AKR* genes to the previously characterized stress inducible alfalfa *MsALR* and the recently characterized *At2g37770* from Arabidospis. Based on homology, chromosomal localization and the mapping of drought stress related QTLs to this chromosomal region we selected 3 *AKR* genes located on chromosome 1, *OsAKR1*, *OsAKR2* and *OsAKR3*. We were interested in the stress inducibility of the selected rice AKRs, so we performed a transcript analysis in response to different stress treatments (ABA, mannitol, H_2O_2, NaCl) applied to rice cell suspensions. As shown by the expression profiles, the *OsAKR1* gene was induced the most by the applied stress stimuli, particularly by ABA (more then 10 fold increase in the transcript level). The other two genes were also stress responsive but showed more modest changes in the transcription levels as compared to *OsAKR1*.

To explain the differences in the gene expression, we performed a motif search in the promoter region of these *AKR* genes by using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html), looking for *cis*-acting regulatory elements, which might help to classify the stress induced transcriptional responses. Our motif search revealed that the *OsAKR1* promoter had the highest number of ABRE and TGACG promoter motifs (linked to ABA and oxidative stress response) among the three rice *AKRs*. Additionally by analyzing the promoters with a newly described enumeration based algorithm we found that *OsAKR1* had the highest number of stress responsive dyad motifs in the promoter compared to the other two genes. Based on the transcript profiling and the abundance of stress responsive promoter elements we selected *OsAKR1* for further analysis.

2) Enzyme kinetic parameters of the recombinant OsAKR1 protein

The enzymatic characterization revealed that the recombinant OsAKR1 accepts a variety of lipid peroxidation and/or glycolysis derived aldehydes as substrates (e.g MDA, MG), along with some sugars also. Among the aldehyde substrates for OsAKR1, MDA and MG are the most important, since these are highly toxic in elevated concentrations and their increase is linked to a variety of

stress processes. The OsAKR1 affinity towards both MG and MDA is represented with Km values that are close to the reported *in vivo* concentrations, predicting that this enzyme could perform its reactive carbolnyl detoxifying role under *in vivo* conditions as well. For the other two AKR proteins we could not show any detectable activity on any of the substrates we tested, probably because of differences in the secondary structure or a very narrow substrate preference which we could not identify.

3) Reactive carbonyls and their role in protein modifications

We could see through the enzyme kinetic parameters, that the recombinant OsAKR1 can effectively reduce toxic carbonyls. Both MDA and MG are protein modifying agents, which exert their action (among other ways) through a mechanism called protein carbonylation. MG is mainly a glycolysis side-product whereas MDA is mostly lipid peroxidation originated. We have shown the negative effects of protein carbonylation by these aldehydes on PRK through immunochemical method and enzyme kinetic approaches. PRK was inactivated to different extents by 5 mM MG and 1 mM MDA, shown by the decrease in the enzymatic activity and the appearance of carbonyl residues, as detected by immunochemical methods. It is worth mentioning that both MG and MDA are substrates of OsAKR1, therefore the overproduction of such an enzyme could be useful in the prevention of reactive carbonyl-induced protein modifications.

4) *In vivo* detoxification properties of OsAKR1 in bacterial and plant systems

In vitro we could demonstrate that the OsAKR1 recombinant protein can successfully metabolize reactive carbonyls such as MDA or MG. The question aroused, whether this function can be demonstrated *in vivo* as well. The overproduction of this enzyme in *Escherichia coli* increased the *in vivo* tolerance of bacterial cells against MG. This indicates the highly conserved function of the AKR proteins, being able to complement the effect of endogenous AKR proteins to fight against the toxic effects of reactive carbonyl stress.

Heterologous overexpression of OsAKR1 in tobacco resulted in better photosynthetic performance after 10 mM MG treatment and heat stress. The transgenic plants also showed less

MDA levels after heat stress and oxidative stress imposed through 100 μM MV treatments. A positive correlation could be established between the expressed OsAKR1 protein levels in the transgenic plants and the degree of stress tolerance. This hints to an existing connection between these two processes. Probably the overexpression of OsAKR1 in the transgenic plants helps in the detoxification of reactive carbonyls generated through the heat treatment and oxidative stress. The transgenic lines had higher total AKR activity then the wild type plants. Surprisingly lower levels of MG were detected in the leaves of the overexpressing plants as compared to the SR1 both before and after heat stress. This is probably due to the constitutive overexpression of the OsAKR1 protein which offers an auxiliary role next to the glyoxalase system in the detoxification of MG.

In this work we demonstrate for the first time that members of the rice *AKR* family could play important role in the carbonyl detoxification-mediated protection of the plant under heat stress. The use of OsAKR1 in transgenic approaches to generate abiotic stress tolerant crop plants would be feasible through their detoxification properties in the reduction of stress-associated reactive carbonyl products. Identification of such genes through genetic, molecular biological methods coupled with the classical breeding approaches aims for the production of stress tolerant crops. In this way the results presented in this work could be of a significant agronomical importance in the production of stress resistant crop genotypes.

Publications related to the thesis

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IF_{2009}: 2.838

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