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

Ph.D. thesis

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List of abbreviations

ABA	abscisic acid
ABRE	ABA-responsive element
AER	alkenal reductase
AKR	aldo-keto reductase
ALDH	aldehyde dehydrogenase
ALR	aldose-aldehyde reductase
APX	ascorbate peroxidase
AS-1	activation sequence-1
BA	benzyl-alcohol
BLAST	Basic Local Alignment Search Tool
bZIP	basic leucine zipper protein
CaMV	Cauliflower Mosaic Virus
CAT	catalase
cDNA	complementary DNA
CE3	coupling element 3
CP47, CP43	chlorophyll binding proteins
DNPH	2,4-dinitrophenylhydrazine
DTT	1, 4-dithiothreitol
DpAR	Digitalis purpurea AKR
DREB2	dehydration responsive element binding protein
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	Expressed Sequence Tag
Fad	fatty acid desaturase
GPX	glutathione peroxidase
GSH	reduced gluthatione
GST	gluthatione-S-transferase
IB	isolation buffer
IPTG	isoproply-β-D-1-thiogalactopyranoside

LB	Luria Bertani
LEA	late embryogenesis abundant
MAP kinase	mitogen-activated protein kinase
MDA	malondialdehyde
MG	methylglyoxal
MS	Murashige Skoog
MV	methylviologen
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NP40	nonyl phenoxylpolyethoxylethanol
OD	optical density
OEC	oxygen evolving complex
OsAKR	*Oryza sativa* aldo-keto reductase
QTL	Quantitative Trait Locus
P5CS	delta-1-pyrroline-5-carboxylate synthetase
PAGE	polyacrylamide-gel electrophoresis
PAM	pulse amplitude modulated
PAR	Photosynthetically Active Radiation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	phosphoenol-pyruvate
PLACE	Plant Cis-acting Regulatory DNA Elements
PMSF	phenylmethanesulfonylfluoride
POD	peroxidase
PRK	phosphoribulokinase
PrxR	peroxiredoxin
PSI	photosystem I
PSII	photosystem II
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene fluoride

qRT-PCR	Quantitative Real-Time PCR
QTL	Quantitative Trait Locus
RCO	reactive carbonyl
RGP	Rice Genome Research Project
RNA	Ribonucleic acid
ROS	reactive oxygen species
RuBisCO	Ribulose-1, 5-Bisphosphate Carboxylase Oxygenase
SDS	Sodium dodecyl sulphate
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TC	tentative consensus
TCA	trichloracetic acid
TF	transcription factor
TFA	trifluoracetic acid
TFBS	transcription factor binding site
TIGR	The Institute for Genome Research

1. INTRODUCTION

1.1 Abiotic stress in plants -a short overview

Plants are sessile organisms, being continuously subjected to environmental changes. In order to survive, they have to cope with a variety of unfavorable conditions that can have negative impact on the bioproductivity. The term abiotic stress is complex. It includes several stresses caused by drastic changes in the environmental conditions, like excessive drought, hypoxia, strong light, extreme temperatures, high salinity, heavy metals etc. Reports predict that these stresses will increase in the future due to the global warming and climate changes (http://www.ipcc.ch). A good example of abiotic stress-induced dramatic loss in the bioproductivity is the 30 % decline in the crop yield following the European heat wave of 2003 (Ciais et al. 2005). As such it is obvious, that a major priority in the plant research is, to decipher the mechanisms behind abiotic stress responses and to find solutions to minimize the losses in the agriculture by increasing the plant resistance through the progresses obtained from the applications of molecular biology.

As a result of abiotic stress, a series of transcriptomic and metabolic changes occur in the plant cells. From the perception of stress signal to the interpretation and conversion to metabolic response, there are several steps: firstly, plant cells pick up the stress signals through various sensors, which are then transduced by several interconnecting signaling pathyways, where secondary messengers, signal transducers and transcriptional factors play important roles. Then, the transduced signal will be converted into a cellular response (gene expression, metabolite synthesis, signaling cascades etc.). The recognition of stress activates signaling pathways that not only transmit informations within individual cells, but throughout the whole plant as well (**Fig. 1**).

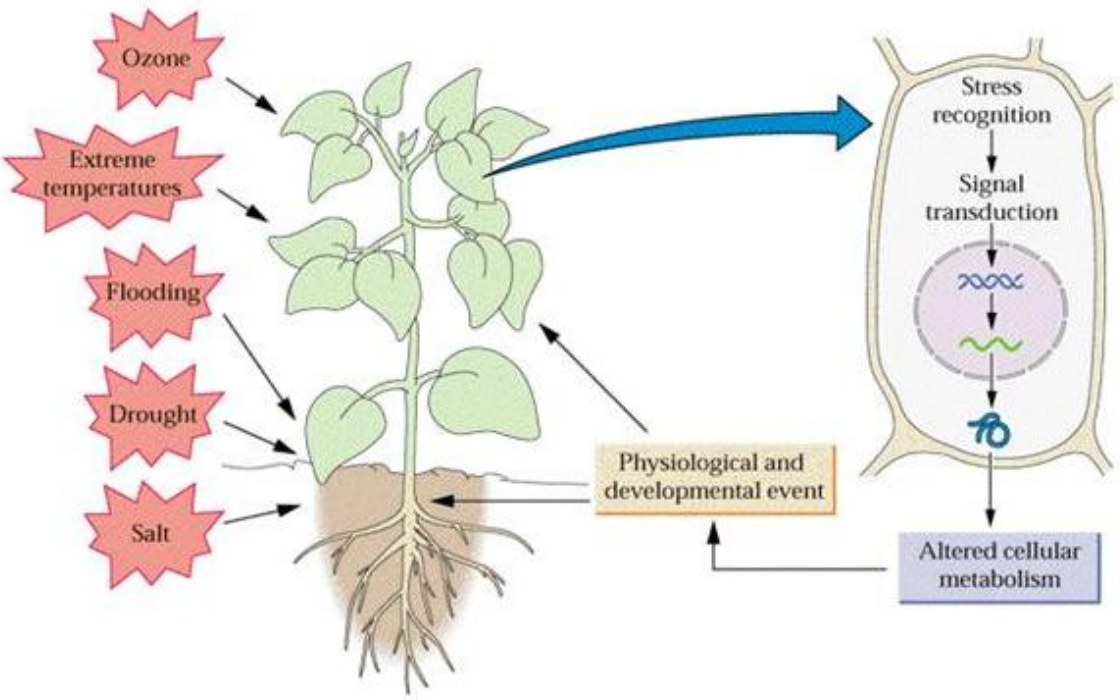


Figure 1. Plant response to abiotic stress (on the cellular- and on the "whole plant" level) (Buchanan 2000). Various environmental signals are picked up by receptors and they are transduced by secondary messengers, to be translated into adaptive transcriptomic and/or metabolic changes. Transduction of environmental signals typically result in altered gene expression on cellular level, which in turn influences the development of the whole plant

Some stress-inducible genes are regulated by multiple stress signals, and their transcription is regulated by transcription factors (TFs) that are induced by stress stimuli. Other stress-inducible genes encode functional proteins that are directly involved in stress tolerance or they encode regulatory proteins which indirectly, as signal transducers form positive and negative feedback loops to regulate stress responses (Hirayama and Shinozaki 2010). The complete genome sequence of *Arabidopsis thaliana*, *Oryza sativa* spp. *japonica* cv. Nipponbare, and other plants has enabled scientist to perform genome-wide gene expression profiling in response to various abiotic stresses (e.g. using AtGenExpress; Kilian et al., 2007). This information has enabled us to get insights into the abiotic stress responses not only on transcriptional level but also in terms of post-transcriptional and post-translational modifications and epigenetic regulations.

1.2. Oxidative stress. ROS formation and their roles in secondary processes

By general definition oxidative stress occurs when there is an imbalance of oxidants (reactive oxygen species (ROS)) and antioxidants. The aerobic lifestyle implies the generation of ROS, which can be considered as unavoidable toxic products of oxygen metabolism. The negative effects of ROS production consists in their interaction with lipids, proteins or nucleic acids and indiscriminate modification, finally leading to the inactivation of cellular functions. Aerobic organisms have evolved antioxidant defenses to protect themselves against this toxicity (Halliwell 1981, Fridovich et al. 1998). In the recent years many studies have emerged, which re-evaluated the concept of oxidative stress. ROS production once considered exclusively harmfull is now being classified as an important component of the plant signalling network. The role of ROS as a signal for gene expression has been evidenced (Desikan et al. 2001, Vanderauwera et al. 2005) and it is also known that ROS is a modulating component in the function of key signaling compunds, such as MAP kinases (Rentel et al. 2004).

Generally, biotic and abiotic stresses increase ROS formation (Apel et al. 2004). **Fig. 2** shows a schematic representation of how different biotic and abiotic stress factors converge into ROS production and thus causing oxidative stress on cellular level, among other toxic effects. The ability of plants to control oxidant level is highly correlated with stress tolerance. An increase in ROS levels can provoke a partial or severe oxidation of cellular components inducing redox status changes (Mittler et al. 2004), so continuous control of ROS is decisive under stress conditions (Meyer et al. 2008). While under non-stress conditions most cellular compartments maintain a reducing environment, stress conditions generally lead to an increase in ROS and induce oxidative stress, which is also defined as a disruption of redox signaling and redox control (Jones, 2006).

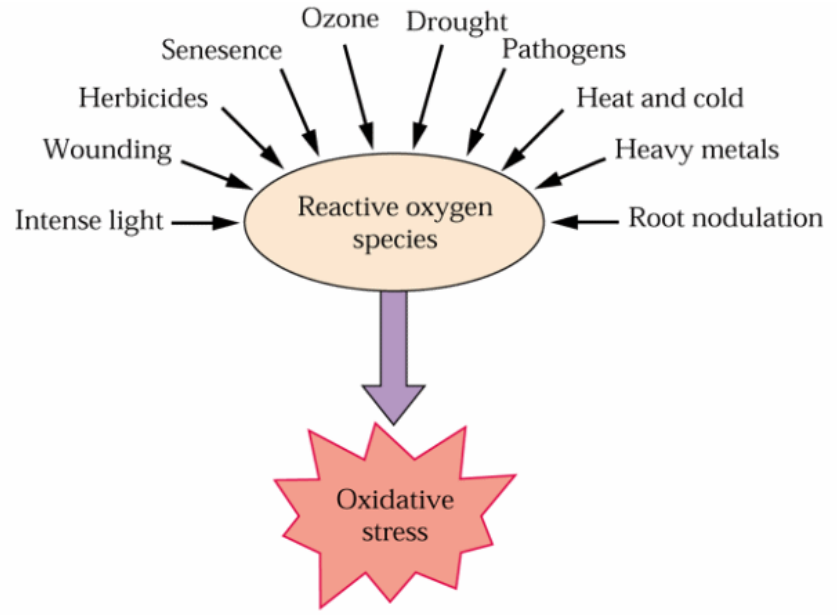


Figure 2. Schematic representation of how different stress factors merge together on the level of ROS production (Buchanan, 2000)

Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and microbodies, are a major source of ROS production in plant cells. In photosynthetic organisms, the chloroplast is the main source of ROS having the capacity to produce high amounts of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), especially during reduced rate of photosynthetic carbon fixation, a situation, which occurs during abiotic stresses (Takahashi and Murata 2008). In addition, chloroplasts can produce singlet oxygen ($^1O_2$) through excited chlorophyll molecules. In non-photosynthetic tissues, mitochondria are the biggest source of ROS, but in a green plant cell, their contribution is considered small in comparison with chloroplasts (Navrot et al. 2007). The redox status of the mitochondrial electron transport chain is an important indicator of the cell energy status and ROS, especially $O_2^{\cdot -}$ from complexes I and III are integral parts of this monitoring system (Taylor et al. 2009).

The third intracellular ROS source is the peroxisomes. They contain several oxidases that produce H_2O_2 and $O_2^{\cdot -}$ as byproducts of the reactions they catalyze. The glycolate oxidase is located in peroxisomes and its function is especially relevant during abiotic stresses. The adaptive response to abiotic stress is often stomatal closure which is followed by a decrease in gas exchange that leads to reduction in carbon dioxide availability for ribulose-1,5-

bisphosphate carboxylase oxygenase (RuBisCO). Consequently photorespiration and H_2O_2 production are increased (Foyer and Noctor 2009).

In addition to these metabolic ROS sources, in the presence of redox-active metals, hydroxyl radicals (OH•) can be formed from H_2O_2 through the Fenton reaction or from H_2O_2 and $O_2^{\cdot-}$ through the Haber–Weiss reaction. The extremely reactive OH• radical can cause extensive oxidative damage in the cell. It is not considered to have signaling function, although the products of its reactions can elicit signaling responses, and cells sequester the catalytic metals to metallochaperones efficiently avoiding OH• formation (Halliwell 2006, Møller et al. 2007).

1.2.1 Reactive carbonyls (RCOs)

The term of carbonyl is reffered to organic compunds which incorporate a functional carbonyl group C=O. Aldehydes and ketons are among these compounds. The carbon atom of carbonyl group has two remaining bonds that are occupied by other substituents. If at least one of these substituents is hydrogen, the compound is an aldehyde. If neither is hydrogen, the compound is a ketone. Reactive carbonyl compounds are formed in a variety of metabolic reactions. Methylglyoxal (MG) for example is formed non-enzymatically from the spontaneous decomposition of triose phosphates, or from the metabolism of threonine and acetol. It is a precursor of glycoxidation products. Other carbonyl species derive from lipid peroxidation (e.g. malondialdehyde (MDA), hydroxynonenal (HNE)). The latter carbonyl products produce MDA- and HNE-protein adducts also termed lypoxidation products (Miyata et al. 1999).

Production of RCOs is reportedly increased by oxidative stress. ROS can directly modify proteins through the oxidation of amino acids (Stadtman and Berlett 1991) or indirectly by the generation of RCO (Miyata et al. 1999)). In the aspect of protein modifications by RCOs we can differentiate two distinct pathways depending on the origin of the carbonyls: Firstly, the non-enzymatic reaction of carbohydrates (or RCOs formed from carbohydrates) with proteins is of great importance. The so-called Maillard reaction is initiated by the attachement of carbohydrates to amino groups of proteins and from reversible products slowly proceeds to the irreversible compounds, leading to covalent crosslinking of the amino

groups of proteins. Basically the end-products of these reaction are called AGEs (advanced glycation end-products). Such products are the carboxymethyl-lysine dimers, imidazolones, methylglyoxal-lysine dimer etc. (Portero-Otin et al. 2003). Secondly, lipid peroxidation originated RCOs such as MDA or, HNE can also react with amino acids of proteins causing protein modification, yielding in lypoxidation products or advanced lypoxidation end products (ALEs). Some ALE's are the for example the MDA-lysine dimer or HNE-lysine dimer. Therefore both AGE's and ALE's are the results of carbonyl-stress products interacting with proteins, the difference being in the pathways of origin for the RCOs (from carbohydrate or lipids). RCOs like glyoxal, MDA and acrolein, are generated by lipid peroxidation of polyunsaturated fatty acids (Esterbauer et al. 1991, Uchida et al. 1998). Others are of glycolytic origin (MG) or can form either via lipid peroxidation or from the oxidation of hydroxi-amino acids (e.g. acrolein) (Miyata et al. 1999).

Generation of RCOs in the cells is unavoidable; therefore effective detoxification mechanisms are necessary to prevent their excessive accumulation. There are several biochemical mechanisms underlying the detoxification of RCOs (Esterbauer et al. 1991; Uchida, 2003). Some well-established routes are: (1) conjugation with GSH catalyzed by the glutathione S-tranferases; (2) oxidation of the aldehyde group to carboxylic acid by either alcohol dehydrogenase or aldehyde dehydrogenase (ALDH); and (3) reduction of the aldehyde or keto moiety to alcohol by aldo-keto reductase (AKR). The mentioned routes here adress the oxidative stress after formation of RCOs although there are other preventive ways (presented in chapter 1.4) which deal with the scavenging of ROS, prior to the generation of RCOs (antioxidant enzymes).

1.2.2. Lipid peroxidation

Some RCOs are the products of ROS accumulation induced degradation of lipids. In this process free radicals steal electrons from the lipids in cell membranes, mostly resulting in cell damage. The common targets for this radical-mediated oxidation are the polyunsaturated fatty acids (PUFA), since they contain multiple double bonds in between methylene (- CH_2) groups that possess especially reactive hydrogens.

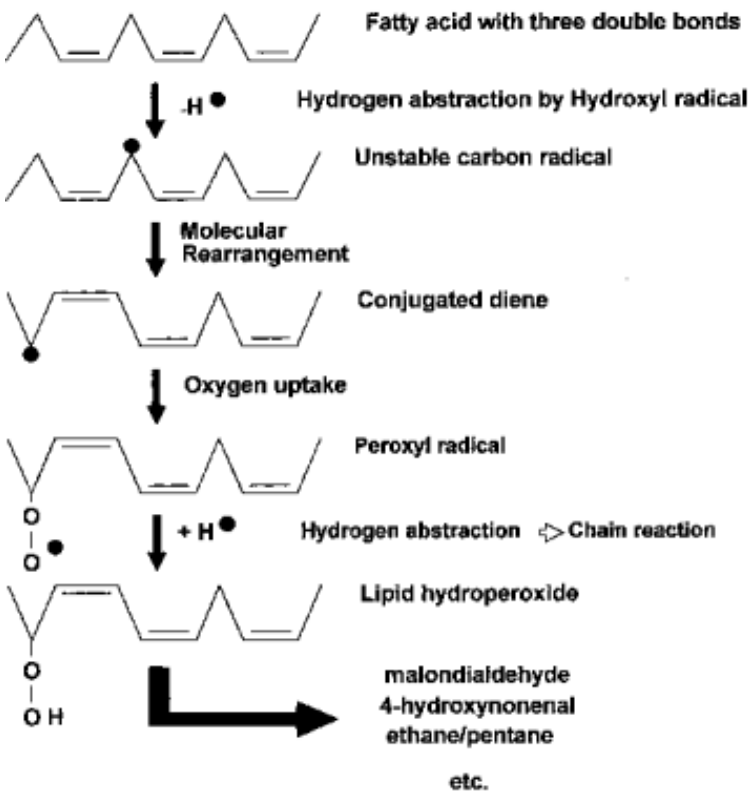


Figure 3. Basic reaction of lipid peroxidation (Young and McEneny (2001)

In the living cells as shown by **Fig. 3** the initiation of the lipid peroxidation chain reaction starts with the formation of fatty acid radicals. The main factors that trigger this event are the ROS, most notably OH• which combines with a hydrogen atom, to make water and a fatty acid radical. The fatty acid radical is not a stable molecule, so it reacts with molecular oxygen creating a peroxyl fatty radical. This creates lipid hydroperoxide which ultimately leads to the formation of reactive carbonyls. When a radical reacts with a non radical, it always produces another radical and therefore this reaction can be termed as chain reaction. One way to stop this chain reaction is when two radicals react and produce a non-radical. This usually happens when the radical concentration is too high, therefore increasing the probability of radical-radical interaction. However, during the evolutionary process living organisms have become well equipped with a series of antioxidant enzymes (SOD, APX, CAT) preventing this event to cause major cellular damages (see 1.4).

1.3. Modification of proteins by reactive carbonyls

Lipid peroxidation products can diffuse across membranes, allowing the reactive aldehyde-containing lipids to covalently modify proteins localized throughout the cell and relatively far away from the initial site of ROS formation. It has been documented that the term protein carbonylation covers the ROS induced post-translational modification of proteins manifesting in the addition of reactive carbonyl groups on certain amino acids. The most reactive and common form of these carbonyl groups exist in aldehydes (Grimsrud et al. 2008). Direct protein carbonylation can be achieved through a variety of reactions; oxidation of amino acid side chains with metals and hydrogen peroxide is known to cause the formation of semialdehyde amino acids, with the majority of these reactions occurring with lysine, arginine, and proline residues (Stadtman and Berlett 1991). Alternatively, protein carbonylation can result from an indirect mechanism involving the hydroxyl radical-mediated oxidation of lipids. Because the side chains of Cys, His, and Lys are often used in catalysis, the most common effect of protein carbonylation is enzyme inactivation.

A common physiological dicarbonyl compound, the MG, is linked to a variety of processes mostly involving protein modifications (cross-linking, protein denaturations) that lead to a number of pathological implications (diabethes, atherosclerosis, (Baynes and Thorpe 2000) etc.). It is mainly derived from glycolytic triose phosphates and is associated with the Maillard reaction, a reaction yielding in the formation of AGE (see 1.2.1) In a report by Park et al. (2003) MG was shown to inactivate in a time and dose dependent manner the bovine gluthatione peroxidase (GPX), a major antioxidant enzyme, by irreversibly modifying the arginine residues located in the gluthatione binding site of GPX. Morover it has also been reported that dicarbonyl compunds (including MG) are able to elevate intracellular peroxide levels (Che et al. 1997) which are responsible for oxidative cellular damage.

As it was mentioned earlier RCOs can form from lipoxidation pathways as well, PUFA's are known to be easily peroxidized in response to oxidative stress (Yamauchi et al. 2008). MDA is a typical end-product from peroxidized PUFA's and it is highly reactive with biomolecules such as proteins and polynucleotides (Esterbauer et al. 1991). The major reactive site of proteins with MDA is the **ε**-amino group of Lys residues, resulting in chemical denaturation of proteins by elimination of the positive charge of Lys (Uchida 2000; Ishii et al.

2008). In plants (especially in the chloroplast membrane) tri-unsaturated fatty acids compose a large percentage of the membrane fatty acids (50-90 %). This guarantees the high fluidity of the thylakoid membrane; however these fatty acids are very sensitive to oxidation. Many ROS are produced in the chloroplast during photosynthesis and therefore the peroxidation products caused by the oxidation of PUFA in the chloroplast membrane can easily yield MDA and might cause protein modifications (Yamauchi et. al 2008). MDA affects PSII functions by modifying proteins such as the oxygen evolving complex (OEC33), CP47 and CP43 in spinach leaves. Their MDA modification causes release of OEC33 from PSII, leading to partial loss of the oxygen-evolving activity (Yamauchi et al. 2010). The above mentioned datas show, that both MDA and MG are reactive carbonyls that can modify and inactivate proteins thus their efficient detoxification is vital in the prevention of cellular damages.

1.4. Protection against ROS by antioxidant enzymes in plants

Aerobic organisms have developed an antioxidant defense system that most probably has co-evolved with the oxidative processes, to disarm the damaging effect of ROS. With a massive supply of oxidases, the plant cells are well-armed for ROS production (Mittler et al. 2004). Major ROS-scavenging enzymes of plants are the superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), GPX and peroxiredoxin (PrxR). The protective enzymes and the antioxidants (ascorbic acid and gluthatione) provide cells with highly competent machinery for detoxifying $O_2^{\cdot -}$ and H_2O_2.

SOD is the major $O_2^{\cdot -}$ scavenging enzyme, although the neutralizing reaction yields H_2O_2. Fortunately the detoxification of H_2O_2 is performed by several antioxidant enzymes: a) the APX together with H_2O_2 and two molecules of ascorbic acid, yields two molecules of monodehydro-ascorbate and H_2O, b) the GPX combines two molecules of reduced gluthatione with H_2O_2 resulting in two H_2O molecules and oxidized gluthatione, c) in the case of PrxR the redox active cystein (the peroxidatic cystein) in the active site is oxidized to a sulfenic acid by the peroxide substrate, d) and the most effective H_2O_2 scavenger is the CAT, able to catalyze the reaction of $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. From the description of this antioxidant system it is clear, that the balance between SODs and the different H_2O_2-scavenging enzymes (APX, GPX, PrxR, CAT) in cells is important for determining the steady-state level of $O_2^{\cdot -}$ and H_2O_2.

Maintaining this balance along with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of the highly toxic OH• radical via the metal-catalyzed Haber–Weiss reaction or the Fenton reaction (Asada and Takahashi 1987, Bowler et al. 1991). OH• is a highly reactive and a very dangerous ROS, since it cannot be eliminated through enzymatic reaction (preventing its formation is the best way of protecting cell compartments against its toxicity). Once formed, due to the very short half-life and high reactivity it is virtually impossible to handle. Antioxidants like gluthatione, vitamin E (á-tocopherol) and effective repair systems are the only means of preventing and/or mending the damage. Reacting with almost every macromolecule (including lipids) is a key player in the lipid peroxidation and the generation of reactive aldehydes.

During abiotic stress, the formation of ROS and consequently the lipid peroxidation process often accelerates. Toxic compunds like HNE, MDA are produced, due to the oxidative damage and the depletion of the above mentioned antioxidant enzyme supplies required for other pathways as well. As such, alternative ways are required to dispose of the reactive peroxidation products. The reduction of the reactive aldehyde groups on these molecules is an important step in clearing HNE and MDA from stressed cells (Simpson et al. 2009), and this role have been attributed to some members of the aldo-keto reductase enzyme family.

1.5. Aldo-keto reductase superfamily (general description)

The aldo–keto reductase (AKR) superfamily comprises a range of generally monomeric 34-37 kDa proteins that are NAD(P)H-dependent and share a common $(\alpha/\hat{a})_8$-barrel structural motif (**Fig. 4**) (Jez et al. 1997)

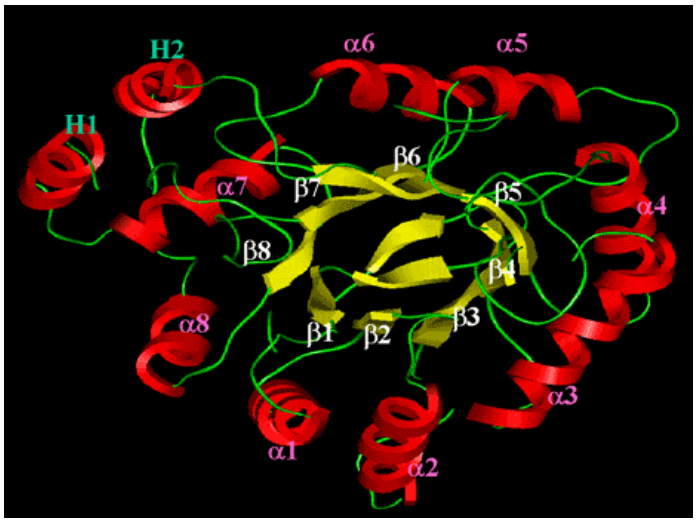


Figure 4. **The $(\alpha/\hat{a})_8$-barrel motif of aldo/keto reductases** (http://www.med.upenn.edu/akr/)

They are widely distributed, from bacteria to human, and typically catalyze the reduction of a number of carbonyl compounds to corresponding alcohols or the reverse oxidation reactions. Substrate specificity is largely determined by three flexible loops (A, B and C) on the surface of the core α/â-barrel, and probably because of the general plasticity of this region, the enzymes typically accept more than one substrate.

A nomenclature system for these enzymes was developed based on amino acid sequence similarity that distinguishes the unique protein isoforms (Jez et al. 1997). The designation "AKR" identifies the protein as a member of the superfamily; a numeric figure designates family, defined by 40 % shared sequence identity; a letter designates subfamily, defined by 60 % shared sequence identity; and numeric figure representing the unique protein sequence. Proteins that share 95 % or higher sequence identity must be demonstrated that they have different functions in order to consider them unique proteins rather then variant alleles of a single protein isoform. A full list of AKRs is publicly available and continuously updated at the AKR website (http://www.med.upenn.edu/akr/)

The wide substrate-specificity of many AKRs has led to difficulties in assigning function. It has been debated in some cases, that their ability to accept multiple substrates is linked to a function in alleviating stress, or that some are capable of detoxifying toxic carbonyls, including both endogenous stress-induced aldehydes, such as HNE, MDA and MG derived from lipid or sugar oxidation, and xenobiotic toxicants (Ellis 2007, Jin and Penning 2007). Relieving the effects of osmotic stress has also been associated with the sugar-

metabolizing aldose reductases (Garcia-Perez et al. 1989). Alternatively, involvement in specific, non-stress associated metabolic processes has been attributed to many AKRs with multiple roles being suggested; e.g. in steroid hormone (Penning et al. 2000, Bauman et al. 2004) and prostaglandin (Matsuura et al. 1998, Desmond et al. 2003) metabolism. These metabolic actions have been connected to different cellular processes in mammals, including cell proliferation and differentiation (Desmond et al. 2003). In these cases, the key factor may not be the *in vitro* substrate preference, but more probably when and where it is expressed and which substrates does it come in contact with, within the relevant cells (Bauman et al. 2004).

1.5.1. Plant AKRs

Extensive investigations about deciphering the role of AKRs in plants are in relatively small numbers. The AKR superfamily contains 15 families, with delineation occurring at the 40 % amino acid identity level. The majority of AKRs studied in plants so far have belonged to the AKR4 family; predominantly to the AKR4C subfamily (delineation of subfamilies occurs at the 60 % identity level). This latter group includes various aldehyde/aldose reductases of largely uncertain function. Still, several AKR4C members have been associated with tolerance of environmental stresses. For example, in barley embryos, synthesis of AKR4C1, the first member of the 4C subfamily, was identified as a protein that conferred desiccation tolerance (Bartels et al. 1991). It was found that synthesis of AKR4C1 is induced by abscisic acid (ABA), a plant hormone that plays an important role in tolerance to dehydration (Hoekstra et al. 2001) and ABA-induced upregulation of AKR4C1 correlates with increased desiccation tolerance (Bartels et al. 1991). Similarly, AKR4C2 and AKR4C3 are upregulated by ABA in bromegrass cell cultures and wild oat seeds, respectively, and are associated with the induction of freezing tolerance (AKR4C2) or have the suggested role of protecting dormant seeds from desiccation (AKR4C3) (Lee and Chen 1993, Li and Foley 1995). Likewise, AKR4C4, cloned from the desiccation-tolerant plant *Xerophyta viscosa*, was identified because it was able to convey tolerance to severe water loss in a desiccation-intolerant mutant *E.coli* strain (Mundree et al. 2000). It has been proposed that AKR4C1–AKR4C4 deliver their desiccation-protecting properties to plant cells through the production of osmolytes (such as sorbitol by reduction of glucose) helping cellular components in the

maintenance of the integrity when the water content is reduced (Bartels et al. 1991, Mundree et al. 2000). However, due to the poor activity of the recombinant enzyme against glucose, a role in clearing different aldehydes has been suggested for AKR4C1 (Roncarati et al. 1995). An alternative role of AKR4C1–AKR4C4 could be the reduction of reactive electrophile species (Farmer and Davoine 2007) such as MG and HNE, which are generated in cells under oxidative stress conditions. Clearing HNE and MG from stressed cells has been suggested as a role for *Medicago sativa* ALR (MsALR) (Oberschall et al. 2000). Both HNE and MG can covalently modify proteins and DNA via their reactive carbonyl groups (Lo et al. 1994, Uchida 2003). Expression of this *MsALR* is induced by various stresses, notably drought, heavy metals, and ABA and overexpression in bacteria produced a protein that could metabolize several substrates, including the stress-induced aldehyde HNE, although the affinity for these substrates (as indicated by a high Km) was relatively low. Ectopic expression of the gene in tobacco resulted in plants with increased drought resistance, suggesting a role in resistance to stress. Similar AKR proteins in *Digitalis purpurea*, DpAR1 and DpAR2, have been shown to have steroid dehydrogenase activity, with a role being postulated in the biosynthesis of cardiac glycosides (Gavidia et al. 2002).

Our studies also support the role of AKRs in alleviating the effects of oxidative stress through their enzymatic properties, by clearing out lipid peroxidation or other toxic metabolic products from the cells. The rice *AKR* genes characterized by us in this study showed abiotic stress inducibility and, the encoded proteins belong to subfamily 4C. We hypothesize based on the data obtained from the detailed characterization of OsAKR1, that these enzymes are important in the detoxification of reactive aldehydes (MDA, MG) generated in under stress conditions (Turóczy et al. 2011).

1.6. Transgenic technology; a usefull tool to improve stress tolerance

Abiotic stresses induce a number of changes in plants; alterations in the gene expression, resulting in increased levels of different proteins or metabolites, some of which actually confer a certain degree of protection to these stresses. By figuring out the mechanisms behind the biochemical and/or molecular changes that occur in response to stress conditions is

a way to successfully obtain stress resistant crops. Classical breeding methods compress the selection of a crop plant with desired agronomical characteristics (good yield parameters, higher stress resistance etc.) and then the attempt of transfering these traits to phylogenetically related species, through a series of cross breeding procedures. Conventional molecular breeding strategies comprise the identification and use of molecular markers (quantitative trait loci (QTLs)) that are involved in stress tolerance. However, the downfall of the above mentioned breeding methods is, that the integration of genomic portions having role in stress tolerance often brings along the integration of agronomically undesirable traits from the donor parents. This is due to the lack of precise knowledge regarding the genes of such a QTL. By such a procedure, a series of selection and crossing attempts are necessary until the right crop candidates with the desirable traits are identified and selected for agronomical benefit. Thus genetically engineering plants proves to be more adequate; it is a faster way to insert beneficial genes then through conventional breeding and it is a more controlled and specific process. In addition, it is the only option where cross breeding is not plausible due to incompatibility barriers, where the genes of interest originate from phylogenetically distant species or non-plant resources (Bhatnagar-Mathur et al. 2008). The transgenic technologies complemented by the classical breeding methods, aim for promising perspectives in creating stress tolerant crops. Through the timeframe of genetic engineering, various transgenic technologies have been implemented to increase stress tolerance in plants. Some of these are summarized in a nutshell bellow.

1.6.1. Transfering single genes

Initially the attempts to develop transgenic plants were limited to the use of single genes to develop stress tolerance. In this way, a gene responsible for a certain function would cause a response (e.g like the modification of a metabolic step) that would confer tolerance a certain abiotic stressor. Stress-induced proteins with known functions such as water channel proteins, detoxification enzymes, key enzymes for osmolyte (proline, betaine, sugars such as trehalose, and polyamines) biosynthesis and transport proteins were the initial targets of plant transformation. The reason behind this approach is that some metabolic traits especially if the pathway involves only a few enzymes can be easier to manipulate then structural and

developmental traits. However it is obvious, that such complex processes like abiotic stress tolerance presumably involves many genes at a time and the single gene transfer cannot be always a good choice to achieve stress tolerance for a broad spectrum of stresses (Bhatnagar-Mathur et al. 2008). Still, here we debate some of the main directions to create stress resistant plants by the transfer of single genes.

1.6.1.1. Genes coding for proteins that detoxify ROS and ROS-generated products

The aerobic lifestyle implies the formation of ROS and environmental stresses increase their production. As such, there is a need to effectively eliminate them. Depending on the nature of ROS some are highly toxic and need to be detoxified rapidly. In order to protect the cells from oxidative injuries plants have developed effective antioxidant machineries that help detoxifying the ROS. A number of transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy. Some of these include the generation of transgenic plants over expressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases (Roxas et al. 1997, Zhu et al. 1999). For instance transgenic tobacco over-expressing SOD in the chloroplast, mitochondria and cytosol have been generated (Bowler et al. 1991; Van Camp et al. 1996) and these have been shown to enhance tolerance to oxidative stress induced by methylviologen (MV) in leaf disc assays. A similar transgenic approach was carried out to obtain tobacco's overexpressing a chloroplast Cu/ZnSOD, which showed significantly improved photosynthetic performance under chilling conditions (Sen Gupta et al. 1993). In some cases however the improved oxidative stress tolerance of the transgenic plants could only be observed when other antioxidant enzymes and substrates were also present (Slooten et al. 1995), suggesting that genotype and the isozyme composition also have a significant effect on the relative tolerance of the transgenic plants to abiotic stress (Rubio et al. 2002). In other cases lower membrane injury was reported in transgenic alfalfa plants overexpressing a MnSOD in their chlorplasts (McKersie et al. 1996).

Overexpression of antioxidant enzymes makes it possible to somehow remedy the oxidative damage already in the initial stages, right after the oxidative burst followed by combined effects of different stress factors. Still ROS mediated cellular damage can also be

reduced if the detoxification strategies target enzymes that perform their scavenging duties on the secondary products of the oxidative stress such as RCOs. There are several reports from this direction. Transgenic tobacco plants overexpressing the *MsALR* showed lower concentrations of reactive aldehydes and higher tolerance against oxidative agents and drought stress (Oberschall et al. 2000). Moreover, recent data revealed that transgenic tobacco's overexpressing OsAKR1 from rice showed also improved tolerance to heat stress and oxidative damage caused by methyviologen (Turóczy et al. 2011).

1.6.1.2. Genetic engineering targeting osmoprotectants

To combat abiotic stress, stress-tolerant plants have evolved effective ways (like to synthesize osmoprotectants) to restore the osmotic balance, a common effect of dehydration-associated abiotic stresses. Defined as osmoregulation, this is a convenient way to cope with stress through the synthesis of osmoprotectants. These are naturally produced by stress-tolerant plants (Cominelli and Tonelli 2010), but most crop plants lack the ability to synthesize them. Therefore genes responsable with the synthesis of osmoprotectants have been incorporated into transgenic plants to confer stress-tolerance (Bhatagnar-Mathur et al. 2008 and Umezawa et al. 2006). Overproduction of compatible solute osmoprotectants such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines), and sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) has been achieved in various target plants. Glycinebetaine in particular has been broadly studied as a compatible solute, both by genetically engineering its biosynthesis in agriculturally important species and by its exogenous application (Chen and Murata 2008). When maize plants were transformed with the *betA* gene from *Escherichia coli (E.coli*) that encodes choline dehydrogenase, they accumulated glycinebetaine in tissues and were more tolerant to drought stress than wild-type plants at different developmental stages. Most importantly they had 10–23 % higher grain yield than that of wild-type plants after three weeks of drought stress (Quan et al. 2004).

In some cases the accumulation of compatible solutes also protects plants against damage by ROS (Bohnert and Shen 1999); in other cases the solutes have chaperone-like activities that protect other proteins maintaining their structure and function (Diamant et al. 2001) and (McNeil et al. 1999).

1.6.1.3. Genes coding for late embryogenesis abundant proteins (LEA) and ionic homeostasis

As suggested by their denomination, the LEA proteins are abundant during late embryogenesis and accumulate during seed desiccation as a response to water stress (Galau et al. 1987). Some of these proteins are important in sequestering ions which are concentrated during cellular dehydration. Hence the overproduction of some LEA proteins could indeed be beneficial upon dessication or ion imbalance. Constitutive overexpression of *HVA1*, a group-3 LEA protein from barley induced tolerance to water deficit and salt stress in transgenic rice plants (Xu et al. 1996). Also, transgenic rice plants expressing a wheat group 2 LEA protein (PMA80) gene or the wheat LEA group 1 protein (PMA1959) gene resulted in increased tolerance to dehydration and salt stress (Cheng et al. 2002).

The ability to maintain or to restore osmotic and ionic homeostasis during abiotic stress is a crucial point in the stress tolerance. The main priority in this direction is to improve salt tolerance of plants through genetic engineering. This has been achieved by increasing the cellular level of some transporter proteins (e.g vacuolar transporters). For example, transgenic melon (Bordás et al. 1997) and tomato (Gisbert et al. 2000) plants expressing the *HAL1* gene showed a certain level of salt tolerance as a result of withholding more K^+ than the control plants under salinity stress.

Through the overexpression of proteins with chaperon functions (proteins which are responsable in the correct refolding of misfolded proteins and/or nucleic acids) it was shown to achieve a broad abiotic stress tolerance. Castiglioni et al. (2008) proved that constitutive expression of bacterial RNA chaperones- *CspA* from *E.coli* and *CspB* from *Bacillus subtilis*-confered abiotic stress tolerance in transgenic Arabidopsis, rice and maize. More importantly this study is backed up by field trials in water limited environments and it was shown to have agronomical significance due to the positive yield benefits resulting in the increase of kernel numbers of maize.

1.6.1.4. Engineering genes of lipid biosynthesis and membrane fluidity

Membrane lipid composition can be decisive factor for survival under abiotic stress conditions. Genes for the lipid biosynthesis and heat-shock proteins are important targets of transgenic research to improve photosynthesis under abiotic stress conditions (Grover and Minhas 2000). For example the survival upon chilling stress greatly depends on the alteration of lipid content of the membranes by increased fatty acid unsaturation. Transgenic tobacco plants with silenced expression of chloroplast *x*3-fatty acid desaturase (Fad7, which synthesises trienoic fatty acids) were able to acclimate to high temperature as compared to the wild type (Murakami et al. 2000). Genetic engineering for increased thermotolerance by enhancing heat shock protein synthesis in plants has been achieved in a number of species (Li et al. 2003; Katiyar-Agarwal et al. 2003).

1.6.2 Manipulating multiple genes

Recently, another promising approach has emerged to improve abiotic stress resistance of plants; the use of genes, that switch on transcription factors and thereby regulate the expression of a whole set of genes related to abiotic stresses. The often complex, genetic and metabolic responses surrounding an abiotic stress response suggest, that stress resistance more likely implies the collaboration of several genes rather then the effect of single, individual genes. Furthermore, several studies highlight well-defined connection between genes or pathways induced by different stresses, suggesting that transcription factors, by turning on/off entire genomic regions might cause multiple stress tolerance, by influencing the transcription of a high number of genes. For example many genes that respond to multiple stresses like dehydration and low temperature at the transcriptional level are also induced by ABA (Mundy and Chua 1988), which protects the cell from dehydration (Dure et al. 1989; Skriver and Mundy 1990).

In order to restore the cellular function and make plants more tolerant to stress, transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels (Bohnert et al. 1995). As mentioned before, to overcome such restrictions, enhancing tolerance towards multiple stresses by a gene encoding a stress

inducible transcription factor that regulates a number of other genes is a promising approach (Yamaguchi-Shinozaki et al. 1994; Chinnusamy et al. 2005). An attractive target category for manipulation and gene regulation is the small group of transcription factors that have been identified to bind to promoter regulatory elements in genes that are regulated by abiotic stresses (Shinozaki and Yamaguchi-Shinozaki 1997; Winicov and Bastola 1997). The transcription factors activate cascades of genes that act together in enhancing tolerance towards multiple stresses. For example the overexpression of DREB2A transcription factor from maize in Arabidopsis resulted in drought and thermo-tolerance and moreover, the transcription profiling of these transgenic plants revealed the upregulation of not only genes coding for LEA proteins, but also genes related to heat shock stress, detoxification and seed maturation (Qin et al. 2007). Similarly the overexpression of drought-responsive transcription factors can lead to the expression of downstream genes and the improvement of abiotic stress tolerance (Zhang et al. 2004). In this aspect, a promising result in the field of crop improvement through the overexpression of transcription factors was provided by Nelson et al. (2007). They identified a nuclear transcription factor which improved the performance of Arabidopsis under drought conditions. Furthermore, they also identified a transcription factor from maize (ZmNF-YB2) orthologous to the one from Arabidopsis, which overexpressed, increased the tolerance of maize plants to drought in water limited environments, based on several stress-related parameters. The importance of this study consists in the monitoring of the “on field performance” of the transgenic plants as opposed to only laboratory conditions.

2. AIMS OF STUDY

With the recent advances made in the functional genomics, rice, as being the world's number 1 food crop, has now become the main focus of genetic engineering as well. Completing of the genome sequencing and the availability of large marker based physical maps enables an easy selection of chromosomal regions containing genes of interest. Considering our research group's long standing interest and experience in single gene transgenic approaches to create abiotic stress tolerant plants through the overexpression of reactive carbonyl scavenging enzymes (e.g. MsALR, by Oberschall et al. 2000) the main aims of our work were the following:

1) Selection and identification of abiotic stress-related *AKR* genes from the newly sequenced *Graminae* rice. To accomplish this we used a wide range of bioinformatical methods such as sequence homology determination with other characterized AKRs, promoter analysis, checking for the presence of stress related QTLs in the selected chromosomal regions etc.

2) Following the gene selection, testing the stress-responsiveness of the candidate genes. Different abiotic stress treatments triggered the accumulation of both the transcripts and AKR protein levels in rice cell suspensions.

3) Enzymatic characterization (substrate specificity, *in vitro* and *in vivo* reactive carbonyl scavenging properties) of the protein coded by the most stress responsive OsAKR1 gene.

4) To show the toxicity of reactive carbonyls and their ability to modify and inactivate functional proteins (Calvin cycle's phosphoribulokinase (PRK))-the reason why the effective detoxification of reactive carbonyls is so important

5) Overproduction of the OsAKR1 protein in tobacco plants and as a long term goal in crop plants to create stress tolerant genotypes.

6) To characterize and to highlight the effects of the overexpression on the improvement of stress tolerance of the transgenic plants (oxidative stress, heat stress) through a series of physiological and biochemical approaches (phothosynthetic measurements backed up by measuring the reactive carbonyl content of the leaves, total AKR activity measurement etc.)

The overproduction of scavenging enzymes taken from crop plants (rice) in a phylogenetically related species (e.g. other cereals) can have a great importance due to possibility of targeted transfer of desirable agronomical traits or increasing stress resistance through manipulating a single metabolic step (contrary to the classical cross-breeding methods, where non desired traits can also be transferred), has higher social acceptance (cis-genis vs. transgenic approaches) and lower chance of foreign gene silencing due to incompatibility barriers (e.g. dicots and monocots).

As mentioned earlier, the final goal of this research is the implementation of a succesfull approach to create cis-genic stress tolerant crops through the overexpression of an enzyme, which can lead to multiple stress tolerance by its action performed mainly in reactive carbonyl detoxification, a process that is the outgrowth for many abiotic stresses. This part of the research still belongs to the near future, although steps were already taken in the analysis of *OsAKR1* overexpressing wheat plants.

3. MATERIALS AND METHODS

3.1. In silico methods (gene selection, homology tree, promoter analysis)

Sequencing of rice genome and the annotation for most of the rice genes, as well as the availability of sequence databases (NCBI, TIGR, etc.) enables an easy access to genetic resources. Based on the available tentative consensus (TC) sequences for *Orzya sativa ssp. japonica* we searched the whole rice genome for genes, assigned as putative *AKRs*. For the identification of rice *AKR* genes by sequence homology with other *AKRs*, two databases were used. The *Oryza sativa* (taxid: 4530) subset of the nucleotide collection (nr/nt) database of the National Center for Biotechnology Information (NCBI) was searched with the Basic Local Alignment Search Tool (BLAST) version 2.2.23 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The second program was selected from the Computational Biology and Functional Genomics Laboratory, DFCI Rice Gene Index webpage (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=rice). Here the target sequence was submitted to the BLASTN program, and searched against the *O. sativa* database. In order to determine the chromosomal localization of the selected genes we used the Rice Genome Research Program (RGP) (http://rgp.dna.affrc.go.jp).

We identified 25 full length *AKR* genes, and we created a homology tree based on the amino acid sequence of all putative rice *AKR* genes, including other previously well characterized plant *AKRs* (e.g. *MsALR*, *DpARs* (*Digitalis purpurea AKRs*)). For both the nucleotide and protein sequence alignments we used the ClustalW program (http://www.ebi.ac.uk/clustalw/) and for the protein secondary structure prediction we used the HNN (Hierarchical Neural Network) method from the NPS@ (Network Protein Sequence Analysis) program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html).

Promoter analysis for the selected genes was performed through PlantCARE (a database for Plant Cis-Regulatory DNA elements http://bioinformatics.psb.ugent.be/webtools/plantcare/html) as follows: From the full length genomic sequence 1.5 kb upstream region from the transcription initiation site (ATG) was designed as promoter. Our primary aim was to identify promoter motifs which might play role in the abiotic stress responses.

3.2. RNA isolation and Quantitative Real Time-PCR experiments

The cDNAs for the Quantitative-PCR reactions were obtained from the total RNA of 100 µM ABA, 5 mM H_2O_2, 100 mM NaCl, 300 mM mannitol treated rice cell suspensions (*Oryza sativa var. japonica* cv. UNGGI-9) by using the TRI Reagent method (Chomczynski and Sacchi, 1987) optimized for plant RNA extraction.The rice cell suspensions were grown in the culture medium according to Li and Murai (1990). The tissues were frozen in liquid nitrogen and stored at – 80 °C before RNA extraction.

RNA was quantified by means of spectrophotometric OD_{260} measurements, and the quality was assessed by calculating the ratio OD_{260}/OD_{280} and by electrophoresis on a 1 % formaldehyde agarose gel followed by ethidium bromide staining. RNA samples were stored at –80 °C. Two-step real-time reverse transcription (RT)-PCR was performed. First-strand cDNA was synthesized from 1 µg of total RNA, using the M-MuLV H minus reverse transcriptase system (Fermentas, Vilnius, Lithuania) with random hexamer primers, according to the manufacturer's instructions. The cDNAs were diluted 1: 3.5 with nuclease-free water. Aliquots of the same cDNA sample were used for all primer sets during real-time PCR, and amplification reactions were performed with all primer sets during the same PCR run. Gene-specific primers were designed for the sequences by the Primer Express Software from Applied Biosystems

We used the following primers: ***OsAKR1*** (Fwd 5'-gacagggaaatttaggagttccgt-3', Rev 5'-caaacattaaatatgcgtaaatttgctaatac-3') ***OsAKR2*** (Fwd 5'-ttagatgcagtcagcttacgttagtg-3', Rev 5'-gctcaagagactcaaacaccataca-3') ***OsAKR3*** (Fwd 5'-atcgaacaggttaagcaaatcaga-3', Rev 5'-ccatcaaaaagctcctcgtaggt-3'). The specific primer pair ***OsACT1*** (Fwd 5'-ctcgtcaggcttagatgtgctagat- 3', Rev 5'-tgaacaatgctgagggattcaa- 3') for the rice *actin 1* gene (accession no. X63830) was used as internal control.

Each oligonucleotide hybridized specifically with cDNAs and produced correctly sized PCR fragments. Specific primers for actin cDNA were used as internal control for real-time PCR in all tested species. Reactions were carried out in a volume of 20 µl containing 150 nM of each primer, 7 µl of cDNA sample (derived from 40 ng of input total RNA) and 10 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as described by

Lendvai et al. (2007). Real-time PCR was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a 96 -well reaction plate, using the parameters recommended by the manufacturer (10 min at 95 °C and 45 cycles of 95 °C for 15 s and 60 °C for 1 min). Each PCR was performed in triplicate, and no template controls were included. The specificity of the amplifications was verified at the end of the PCR run, using ABI Prism Dissociation Curve Analysis Software. The efficiencies of the amplicons were analysed by a standard curve method. Two independent experiments were performed, including reverse transcription and real-time PCR steps. The $2^{-\Delta\Delta CT}$ method was used to analyse the real-time PCR data (Livak and Schmittgen, 2001). The expression of the examined genes was normalized to the endogenous control, in this case to the rice actin genes, to compare the expression levels relative to a calibrator sample. The expression level was also normalized with the aid of an absolute quantification method based on the standard curve, using a known amount of template DNA during real-time PCR detection (Rutledge and Cote, 2003). The relative expression values matched the expression values observed during use of the $2^{-\Delta\Delta CT}$ method.

3.3. Cloning procedures

Based on the qRT-PCR results we selected the product of the *OsAKR1* gene for further analysis and amplified the full-lenght coding region with the following primers containing *EcoRI* and *SalI* restriction sites (bold and underlined in primer sequence):

OsAKR1F-5'-ac**gaattc**atggcgaagcacttcgtgctc-3'

OsAKR1R-5'-ac**gtcgac**tgattggcactgacggaactcc-3'

The *OsAKR1* cDNA was cloned in frame with N-terminal glutathion-S-transferase at *EcoRI* and *SalI* sites in the pGEX-4T1 expression vector (Amersham-Pharmacia), and *E. coli* BL21-(DE3) strain was transformed with the selected construct. Plasmid DNA was purified according to a standard alkali lysis protocol described by (Birnboim et al. 1979) and the nucleotide sequence of the *OsAKR1* clone was determined by the ABI 3100 Genetic Analyzer from Applied Biosystem.

3.4. Tobacco transformation

The *OsAKR1* cDNA was cloned into the derivative of pCAMBIA1200 binary vector (source: Arabidopsis Biological Resource Center, The Ohio State University) under the regulation of the strong CaMV35S promoter. PCR amplified full length sequence of *OsAKR1*, *OsAKR2* and *OsAKR3* was digested with EcoRI and SalI enzymes and cloned in frame in the plant expression vector. After sequencing of the clones, *Agrobacterium tumefaciens* strain EHA 105 was transformed with the right constructs. Tobacco (*Nicotiana tabacum* cv. Petit Havanna line SR1) plants were infected and co-cultivated with the *Agrobacterium* cell suspension, and the regenerated plantlets were transferred and selected on Murashige Skoog (MS) media (Murashige and Skoog 1962) supplemented with phosphynotrycine, according to the method described by (Horsch et al. 1985). Presence of the OsAKR1 protein in the transgenic plants was verified by Western hybridization and from the original 17 independent transformant lines three were selected for further analysis. Both SR1 and the *OsAKR1* overexpressing plants were grown in the greenhouse in 15-cm-diameter pots on a mixture of soil and vermiculite; under natural light conditions corresponding to 120-200 µmol/ m^2 s^1 photosynthetically active radiation (PAR).

3.5. Western hybridization

Two leaf discs from each plant (approximately 50 mg fresh weight) were mechanically pulverized in liquid nitrogen, and after adding 200 µl of isolation buffer (IB) (25 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 60 mM β-glycerophosphate, 2 mM 1,4-dithiothreitol (DTT), 0.1 % nonyl-phenoxyl-polyethoxylethanol (NP 40), 1 mM NaF, 1 mM phenyl-methane-sulfonylfluoride (PMSF)), the leaf homogenates were thoroughly vortexed. After a 10 min 4 °C centrifugation at 13000 g, the supernatant was collected, and the concentration of 2 µl was measured by the method of (Bradford 1976). Same amount of protein (10 µg) from each sample, together with the one isolated from the untransformed control was loaded on a 10 % SDS poliacrylamyde gel, and then blotted onto Immobilon-P PVDF membrane manufactured by Millipore. We used the α-MsALR antibody and the

peroxidase (POD) conjugated anti-rabbit IgG antiserum as described earlier (Oberschall et al. 2000).

3.6. Protein expression

The BL21-(DE3) strain was used for the glutathione-S-transferase (GST) fusion protein expression and purification, after 16 h induction with 0.1 mM isoproply-β-D-1-thiogalactopyranoside (IPTG) at 22 ºC. Samples were centrifuged (10 min 4000 g) and the bacterial cells were washed with ice cold 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Following centrifugation the bacterial pellet was resuspended in 1x PBS containing 2 mM PMSF, 1 mM DTT and lysosyme at final concentration of 100 µg/ ml. After this, cells were sonicated, and Triton X-100 was added in the solution to a final concentration of 0.25 % (v/v). The lysate was placed on shaker for 20 min on RT. Following a 15 min centrifugation on 10.000 g the clear supernatant was used for affinity purification. All steps were done at 4 °C, unless otherwise stated. The affinity purification of GST-fusion proteins was carried out using the Glutathione Sepharose purification protocol according to the manufacturer's instructions (Amersham-Pharmacia Biotech AB). The protein concentrations were determined by the method of (Bradford 1976). Purity of the GST-fusion proteins was checked by PAGE on 10 % SDS-polyacrylamide gels.

3.7. Enzyme kinetics

The *in vitro* enzyme activity measurements for the GST-OsAKR1 were performed in 1 ml reaction volume containing 0.1 M sodium phosphate buffer, pH 7, 0.1 mM NADPH and the appropriate substrate (Vander Jagt et al. 1992). All these reagents were purchased from Sigma. MDA was prepared by acidic hydrolysis from malondialdehyde bis (dimethylacetal) according to (Kikugawa et al. 1980). Briefly, 10 mmol of malondialdehyde bis (dimethylacetal) was mixed with 0.9 ml of 1 M HCl and the heterogenous mixture was shaken at 40 °C until it became homogenous. The solution was made up to 10 ml with water and the

acidic solution was incubated at 37 °C for 1 hr for use (1 M MDA solution). Before using the MDA stock, the pH of the solution was adjusted to 7 with 1 M Na_3HPO_4.

The AKR activity of the leaf extracts was measured using the same buffer and NADPH concentration as described above, with 2 mM MG as the standard substrate. Around 0.4 g leaf material was homogenized under liquid nitrogen with 400 µl of IB. The thawed homogenate was centrifuged for 10 min at 13.000 g. The supernatant was saturated with ammonium sulphate to 25 %. After centrifugation, the concentration of the ammonium sulphate was raised to 50 % and further to 80 % in the supernatant. The proteins precipitated in this manner were dissolved in small amount of 0.1 M sodium phosphate buffer, and were dialysed. All steps were done at 0- 4 °C. Presence of the AKR proteins in the obtained fractions was checked through Western blotting and the 25-50 % fraction was used to assay the AKR activity.

Reactions were monitored at 340 nm, 25 °C, using a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer. For GST-OsAKR1 the decrease in the NADPH concentration was monitored for 1 min, and for the leaf extract 2 min. Reaction was started with the addition of NADPH, only in the case of leaf extracts it was started by the addition of substrate. The enzyme kinetic parameters for the recombinant OsAKR1 (Km, kcat, kcat/Km values) were calculated through the Lineweaver-Burk plot, using the online available BioData Fit 1.02 EC50 software from Chang Biosciences (http://www.changbioscience.com/stat/ec50.html).

3.8. Stress treatments on rice cell suspensions

Rice (*Oryza sativa var. japonica* cv. UNGGI-9) cell suspensions were treated separately with 20 mM benzylalcohol (BA), 100 µM ABA and 5 mM H_2O_2, 42 °C heat treatment. Samples were collected at different timepoints and cells were frozen in liquid nitrogen. For the protein isolation we pulverized approximately 0.5 g frozen cell suspension in 400 µl of IB, and after a 10 min centrifugation on 10.000 g we measured the protein concentration of 2 µl with the Bredford method. Equal amount of protein (10 µg) was loaded on 10 % SDS-polyacrylamide gel, and blotted onto Immobilon-P PVDF membrane. The Western hybridization was performed with the same antibodies as described in *3.5.*

3.9. Methylglyoxal treatments

E. coli BL21-(DE3) cells harbouring the *pGEX4T-1::OsAKR1* expression construct were grown at 37 ºC in 2 YT liquid medium to early exponential phase (OD_{600}= 0.4) and the production of the GST-fusion protein was induced by the addition of 0.2 mM IPTG. After 60 min. 25 ml aliquots were exposed to a 2 mM concentration of methylglyoxal, and OD_{600} was determined for 1 ml aliquots at every 30 min for 2 hours. Before the treatment, cells were harvested from 1 ml aliquots each and the presence of the induced fusion protein was verified by Western blotting, using the α-MsALR antibody described by (Oberschall et al. 2000). From the obtained values we calculated the treated/control ratios and compared them with the negative control (BL21 (DE3) cells producing the GST protein only). MG treatment on the wild type and transgenic tobacco plants was carried out in the following manner: Leaf discs from 6 week old tobacco plants were placed on filter papers soaked with 10 mM MG solution, and kept in glass dishes under a constant illumination of 50 μmol m^{-2} s^{-1} PAR. Photochemical yield on the samples was measured after 18 and respectively 24 hrs, as described in detail in *3.12* section.

3.10. Methylglyoxal measurement

The method was performed as described by (Yadav et. al 2005) with slight modifications. Different concentrations (10, 25, 50, 75, 100 and 200 μM) of pure MG (Sigma) were derivatized with 1, 2-diaminobenzene and the absorbance of the resulting derivative was read by Shimadzu (Kyoto, Japan) UV-160 spectrophotometer. One milliliter of total reaction mixture contained: MG solution in different concentrations, 250 μl of 7.2 mM 1, 2 - diaminobenzene, 100 μl of 5 M perchloric acid, and double-distilled H_2O. In the control, MG was not added to the reaction mixture.

About 0.4 g tissue was extracted in 4 ml of 0.5 M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4 °C at 10.000 g for 10 min. A colored supernatant was obtained in some plant extracts that was decolorized by adding charcoal (10 mg/mL), kept for 15 min at room temperature and centrifuged at 11.000 g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated

solution of potassium carbonate at room temperature and centrifuged again at 11.000 g for 10 min. Neutralized supernatant was used for MG estimation.

3.10.1. Methylglyoxal assay

In a total volume of 1 ml, 250 µl of 7.2 mM 1,2-diaminobenzene, 100 µl of 5 M perchloric acid, and 650 µl of the neutralized supernatant were added in that order. The absorbance of the derivatized MG was read starting immediately at a broad spectrum range (200–500 nm) for 15 cycles of 1 min interval each. In this spectrum a peak height was observed at 332 nm corresponding to derivatized MG. The absorbance at 332 nm was read at exactly the same timepoint in all the measurements in order to ensure the reliability of the data. The concentration was calculated based on the standard curve and expressed in µM.

3.11. In vitro protein modification experiments by MG and MDA

3.11.1 Phosphoribulokinase assay

Arabidopsis thaliana PRK (*At1g32060*) full-length coding sequence was PCR amplified and cloned in frame into *NcoI BamHI* sites of pET28A (+) (Amersham-Pharmacia) using the following primers described by Marri et al. (2005): PRK_Fwd 5'-AGAAA**CCATGG**TGATCGGAC-3' and PRK_Rev 5'-TT**GGATCC**GTTTGTTTTAGGC-3' (restriction sites bolded and underlined in the primer's sequence). *E.coli* BL21-Gold strain was transformed with the construct and recombinant PRK-His protein was purified on 22 ºC induction for 15 hrs with 0.4 mM IPTG. For the purification a Ni-affinity column was used and for the elution different concentrations of imidazole solutions (50 mM, 100 mM, 150 mM, 200 mM, 250 mM). Following elution, the imidazole was removed from the protein solutions, the samples were desalted (PD-10 columns GE-Healthcare) and buffer was changed to assay the enzymatic activity. Enzyme activity was assayed in a buffer (Buffer A) containing: 50 mM TRIS-HCl pH 7.5, 1 mM EDTA, 40 mM KCl, 10 mM $MgCl_2$, and 5 mM DTT (for full activation of the enzyme). The total 1 ml of the reaction mix contained Buffer A (variable

volumes), recombinant PRK-His protein, 2 mM ATP, 2.5 mM PEP, 5 U/ml pyruvate kinase, 6 U/ml lactate dehydrogenase, 0.2 mM NADH, 0.5 mM ribulose phosphate (Marri et al. 2005). Typically 4 μg of PRK-HIS was used for one measurement. Reaction rate was monitored by the oxidation of NADH at 340 nm in a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer. Before each assay the 5 mM DTT (used to fully reduce the PRK) was removed from the system by gel filtration in order to prevent the quenching of the aldehyde effect.

3.11.2 Aldehyde treatments and immunochemical detection of protein carbonyls

Aldehyde treatments were done in the same buffer used for the activity measurement, at 37 ºC. MDA was prepared by acidic hydrolysis from malondialdehyde bis (dimethylacetal) (Kikugawa et al. 1980). MG was purchased from Sigma-Aldrich (St. Louis, MO). Following the incubation the unreacted aldehydes were removed from the mix by gel filtration. Afterwards PRK activity was assayed as decribed above.

2, 4-Dinitrophenylhydrazine (DNPH) was used to detect the extent of protein carbonylation in reactive aldehyde modified PRK. The modified protein samples were mixed with ½ volumes of 24 % SDS and ½ volumes of 20 mM DNPH 20 % trifluoric acid (TFA) solution. The samples were shaken on room temperature for 15 minutes, then the reaction was stopped by the addition of 1 volume of STOP solution (30 % glycerol, 2 M TRIS). The samples were then loaded on 10 % SDS poliacrylamyde gel, and then blotted onto Immobilon-P PVDF membrane manufactured by Millipore (Billerica, MA). The α-DNP primary antibody (Sigma-Aldrich, D9656) and the horseradish peroxidase conjugated anti-rabbit IgG antiserum as secondary antibody were used to detect the carbonylated products.

3.12. Heat stress treatments

Fully expanded leaves (3rd from the top), taken from 6 week-old, T2 generation tobacco plants (wild type and transgenic) were briefly washed in deionized water and 12 mm leaf discs were punched out using a cork borer. Three lines were selected (B1/1, D3/2, D3/3), and 6 leaf discs from each plant were measured in one experiment. The leaf discs from each

plant were placed in perforated 24-well ELISA plates (Greiner Bio One) and floated in a 44 °C water bath for 50 minutes, under a constant illumination of 50 μmol/m^2 s^1 photosynthetically active radiation (PAR). After this treatment, the plates were placed in room temperature distilled water and left to recover for 15 min under the same PAR (in the case of MG and MDA measurement following heat stress, the leaves were left to recover in distilled water for 24 or 48 hrs) After recovery, the photochemical yield of PS II electron transport (Y) of each leaf disc was calculated from variable chlorophyll fluorescence parameters, measured with a Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany), as $Y = (F_m' - F') / F_m'$ according to (Schreiber et al. 1986). F' and F_m' are fluorescence intensities measured before and after a saturating (8000 μmol m^{-2} s^{-1}) light pulse applied at 50 μmol/m^2 s^1 PAR. Statistical analysis was carried out using standard t-test from the Microcal Origin (Version 6.0) program (Microcal Software, Inc.).

3.13. Leaf disc oxidative stress assay

Leaf discs from 6 weeks old plants (3rd leaf from the top) were floated on either 1 μM or 2 μM MV in 24-well ELISA plates. After 2 hours of dark adaptation, allowing the herbicide to penetrate the leaves, the plates were placed under 50 μmol/m^2 s PAR for 20 hours. Following this treatment, lipid peroxidation products were measured and expressed as 2-thiobarbituric acid-reactive substances (TBARS) (Chaoui et al. 1997). Two leaf discs were ground in 1 ml 0, 25 % (w/v) 2-thiobarbituric acid (TBA) and in 10 % (w/v) trichloracetic acid (TCA). After heating the solution at 85 °C for 45 min, the mixture was quickly cooled in an ice-bath, and centrifuged for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the value recorded at 600 nm. Blunt samples lacking plant material were treated similarly. The TBARS were quantified using $1,55x10^5$ M^{-1} cm^{-1} as extinction coefficient for malondialdehyde (MDA)-TBA adduct.

4. RESULTS

4.1. Database search of rice AKR genes. Phylogenetic tree, classification, homologies, secondary structure predictions

As a recently sequenced monocot crop plant, rice has come in the spotlight of functional genomics. Using the available information of the rice genome, our work started by searching for highly homologous rice *AKR* genes with the previously characterized stress inducible alfalfa *MsALR* (Oberschall et al. 2000) and the recently characterized *At2g37770* from Arabidospis (Simpson el al. 2009). As a result we have identified a chromosomal region abundant in aldo-keto reductases (consisting of three highly homologous *AKR* genes) on the 1st chromosome of *Oryza sativa ssp japonica.* The *AKR* genes on this particular region showed high homology with *MsALR* (**Fig. 5a**) and were located in the close vicinity of the Δ^1-pyrroline-5-carboxylate synthetase (*OsP5CS2)* gene that is well known for abiotic stress responsiveness (**Fig. 5b**).

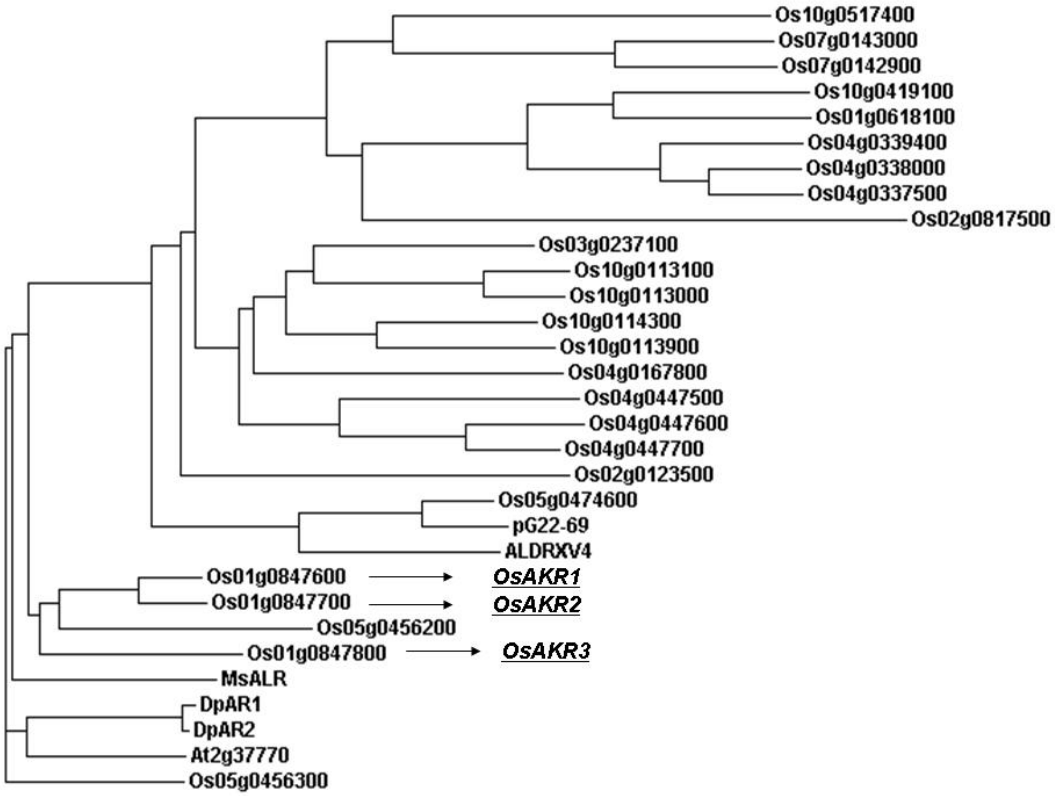


Figure 5a. **Phylogenetic tree based on the amino acid sequence of all the identified putative rice AKRs in comparison with several other characterized plant AKRs (barley pG22-69: Bartels et. al 1991, MsALR: Oberschall et. al 2000, DpAR1, DpAR2: Gavidia et. al 2002, ALDRXV4: Mundree et. al 2000, At2g37770: Simpson et al. 2009).** The multiple sequences were aligned using CLUSTALW program. All the rice genes are represented by their genomic identifiers and those which are the purpose of the present study are highlighted with italics and underlined.

The purpose of the phylogenetic tree was also to identify all the annotated rice *AKR* genes up till now and to group them based on the existing homology. In order to illustrate the similarities between rice and *AKR* genes from other plants species we have included some of the well characterized *AKR* genes from different plant species in the phylogenetic tree. In terms of sequence similarities and functional characteristics the selected genes for our study (highlited with italics on **Fig. 5a**) belong to the AKR4C subfamily of aldo-keto reductases. We identified 25 putative rice *AKR* genes with different chromosomal localizations

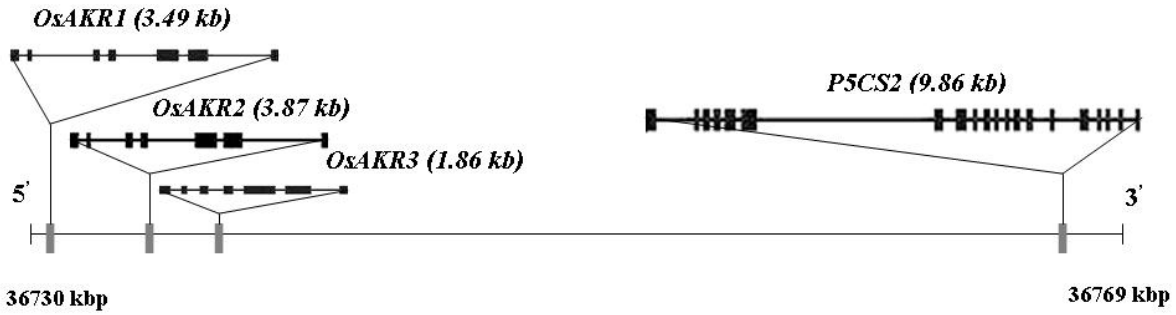


Figure 5b. Schematic representation of the *AKR* rich region on the rice *Chr1* in the close vicinity of the delta-1-pyrroline-5-carboxylate synthetase (*P5CS2*) gene. The genes (*OsAKR1: Os01g0847600, OsAKR2: Os01g0847700, OsAKR3: Os01g0847800* and *P5CS2: Os01g0848200)* are represented according to their localization on the chromosome. Black rectangles stand for the exons, and the size of genomic regions presented in the figure is approximate. Full genomic sequence for this section of Chr1 was obtained from the NCBI Genebank (http://www.ncbi.nlm.nih.gov) and based on the coding sequences located in the full genomic sequence the exon-intron boundaries were determined.

The presence of the AKR rich region on the *Chr1* is highlighted on **Fig. 5b**. Based on the full genomic- and the coding sequence, we determined the exon intron boundaries for both the rice *AKR* genes as well as for the *P5CS2* gene, a close neighbour of the rice *AKRs*. In this schematic representation we can see, that the three genes have a highly similar exon-intron structure, especially the *OsAKR1* and *OsAKR2*. Their chromosomal position (situated virtually next to each other) and the high sequence similarity hints to the fact, that they might be the results of a possible gene duplication. The encoded AKR proteins have an equally similar, conserved structure, particularly OsAKR1 and OsAKR2 with more then 85 % identity (**Fig. 5c**).

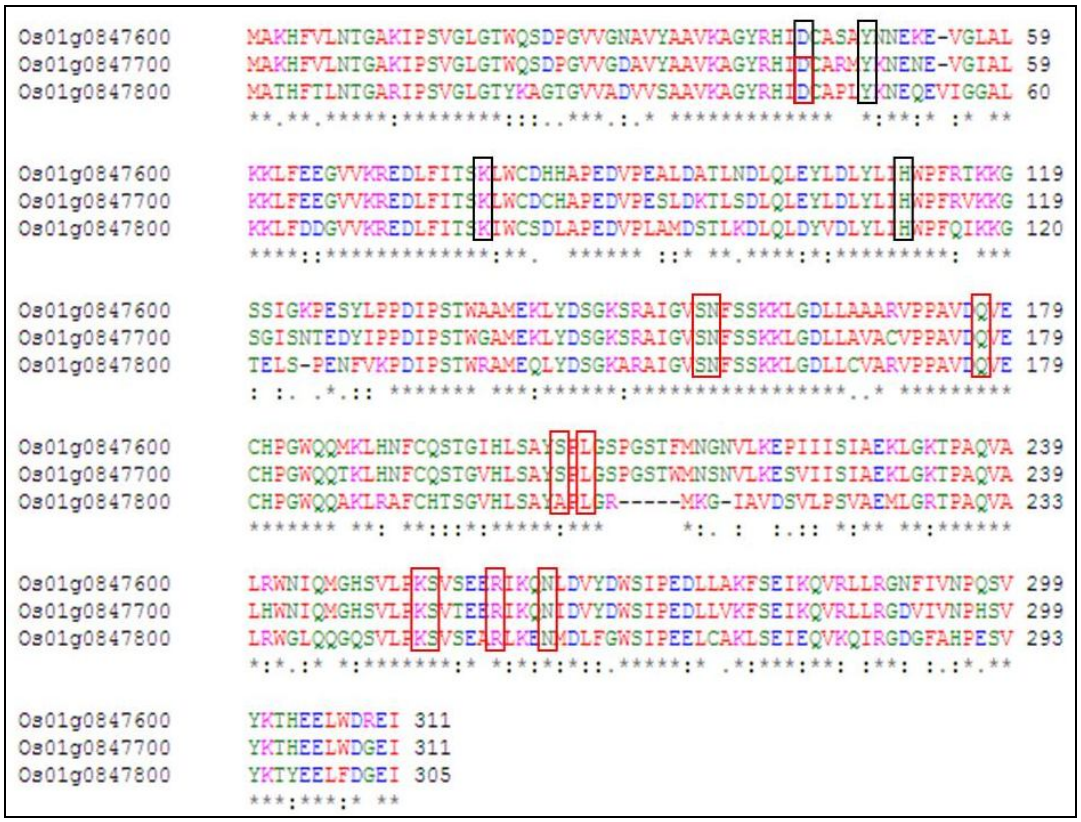


Figure 5c. Alignment based on the amino acid sequences of the *Os01g0847600* (OsAKR1), *Os01g0847700* (OsAKR2) *Os01g0847800* (OsAKR3). Sequences were aligned using ClustalW. (*) denotes identical amino acids in all 3 sequences. The conserved amino acids constituting the catalytic tetrad (namely: Asp44, Tyr49, Lys78, and His111) are highlighted with black bars, whereas the residues for NADPH binding (Asp44, Ser154, Asn155, Gln176,Ser203, Leu205, Lys250, Ser251, Arg256, and Asn260) (Morita et al. 2007) are marked with red bars. (*) indicate identical residues in all sequences, (:) conserved substitutions, (.) semi-conserved substitutions

Based on the sequence alignment we can notice that the amino acids of the conserved catalytic tetrad are identical in all three proteins. These, along with the NADPH binding domain are the active site residues which are conserved in the AKR superfamily enzymes. In the case of the NADPH binding domain all amino acids are identical in the three sequences, except one non-conserved substitution in the OsAKR3 (Ser203 into Ala203). Despite this substitution we can easily notice the high homology between the three sequences, especially between OsAKR1 and OsAKR2. Similarities can be observed in the predicted secondary structrue as well; however important differences can also be noticed (**Fig. 5d**).

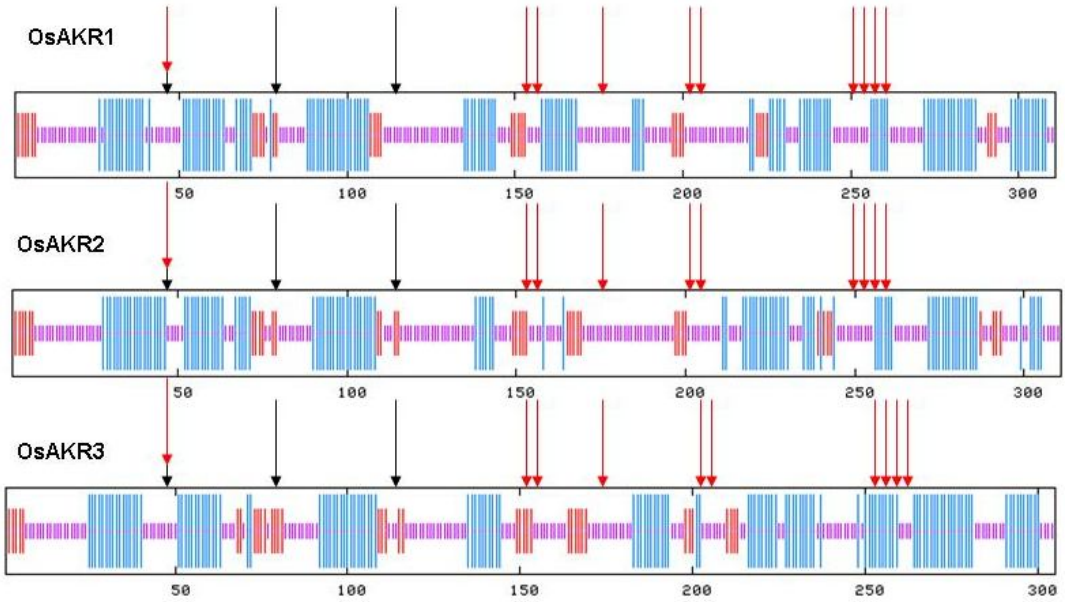


Figure 5d. Secondary structure prediction of the three OsAKR proteins by using the HNN Secondary Structure Prediction Method from the Network Protein Sequence Analysis (NPS@). Light blue colour denotes the presence of alpha helixes and pink colour represents the extended strands in the secondary structure. Black arrows indicate the position of aminoacids that make up the catalytic tetrad (Asp44, Tyr49, Lys78, and His111) and red arrows are indicating the residues responsible for NADPH binding (Asp44, Ser154, Asn155, Gln176, Ser203, Leu205, Lys250, Ser251, Arg256, and Asn260). The position of the arrows is approximate.

The differences in the secondary structures may affect the functional characteristics and the substrate specificity of these proteins. We can see differences in the position of alpha helixes in the amino acids constituting the catalytic tetrade and NADPH binding residues as well. In the presented sequence alignment, the OsAKR1 and OsAKR2 have considerably high similarity on the level of secondary structure also, although significant differences can also be observed in the protein sequence positioned between amino acids 200 to 250 or even in the region 150 to 200 in all the three proteins. The number and position of the alpha helixes are highly different in these regions. These differences in the secondary structure might attribute different functional properties and substrate preferance to these enzymes (see Discussion).

4.2. Promoter analysis and expression profiles of the OsAKR genes following different abiotic stresses

The involvement of *OsAKR* genes in the abiotic stress responses was of our great interest, since the stress inducibility of a gene forecasts a putative role in the mechanisms surrounding the stress tolerance. In order to find evidence, we verified the accumulation of selected transcripts during salt, ABA, osmotic and oxidative stress treatments. The expression profile revealed that the *OsAKR1* gene was induced the most by the applied stress stimuli. The activation of this gene was rapidly triggered by ABA and hydrogen-peroxide, already after 20 min of treatment, followed later by a peak value of 12-16 fold increase in the transcript level at 8 hrs (**Fig. 6a**). Salt and mannitol treatments were found to be less effective and caused a delayed stimulation in the expression of this gene variant. The transcript level of the *OsAKR2* gene was also induced primarily by H_2O_2 treatment, reaching a 4-fold increase of transcription, with an early maximum at 20-60 min (**Fig. 6b**). In contrary, the *OsAKR3* gene showed a late, but significant response to the stress signal generated by ABA with a 6-fold increase in transcript level after 24 hrs (**Fig. 6c**).

Fig 6a

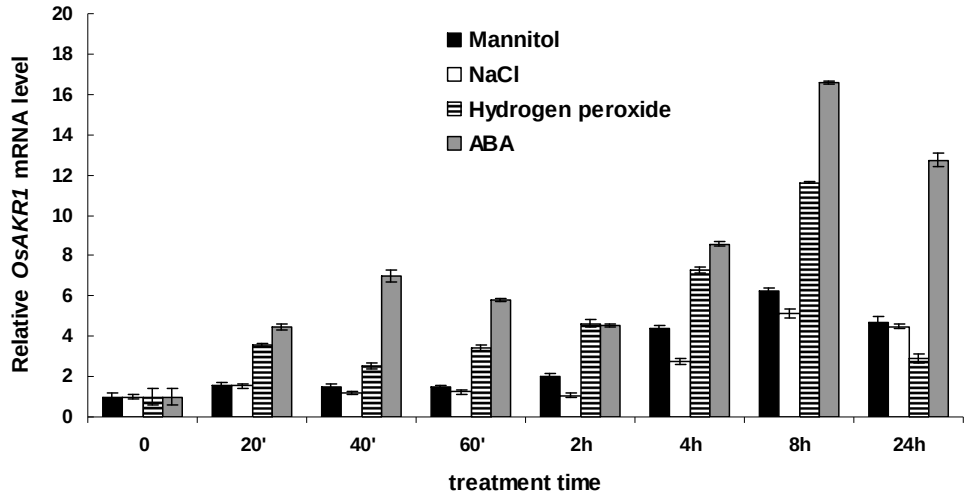


Fig 6b

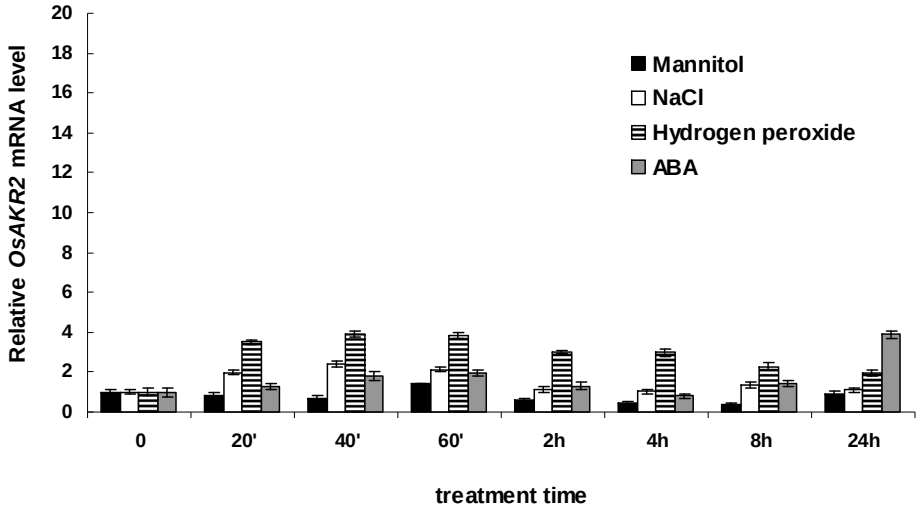


Fig 6c

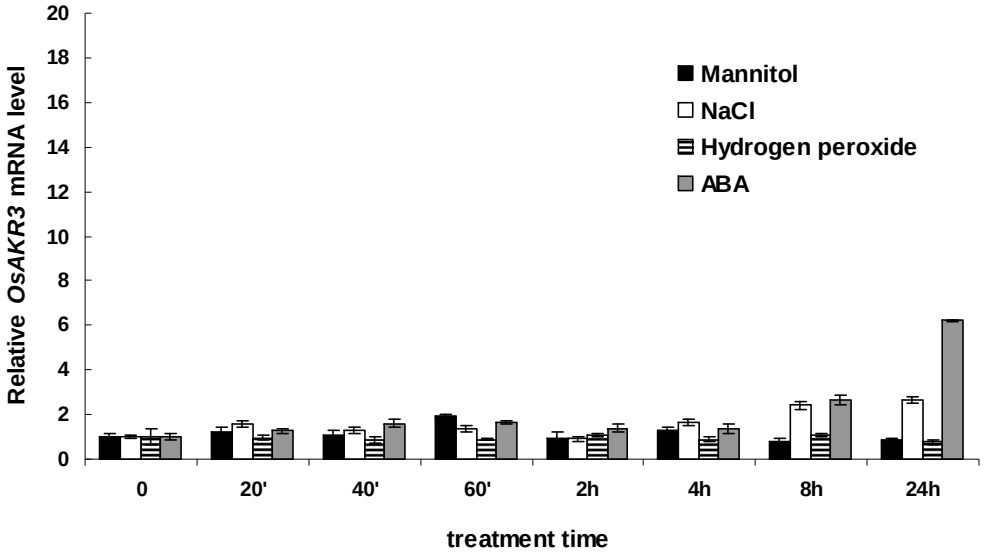


Figure 6a, 6b, 6c. Induction of *OsAKR* gene expression shows gene- and stress-specific features in rice cell suspension. Values on the X axis represent the time points of the treatment. The bars indicate the type of treatment: *black* - 300 mM mannitol, *white*-100 mM NaCl, *stripes* - 5mM H_2O_2, *gray* - 100 μM ABA. Results represent the average of 3 independent experiments.

In order to get a deeper insight to the mechanism of the gene expression induction 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. The differences in the transcriptional induction might be attributed to the quantity and quality of the cis-acting regulatory elements present in the promoters (**Fig 7**).

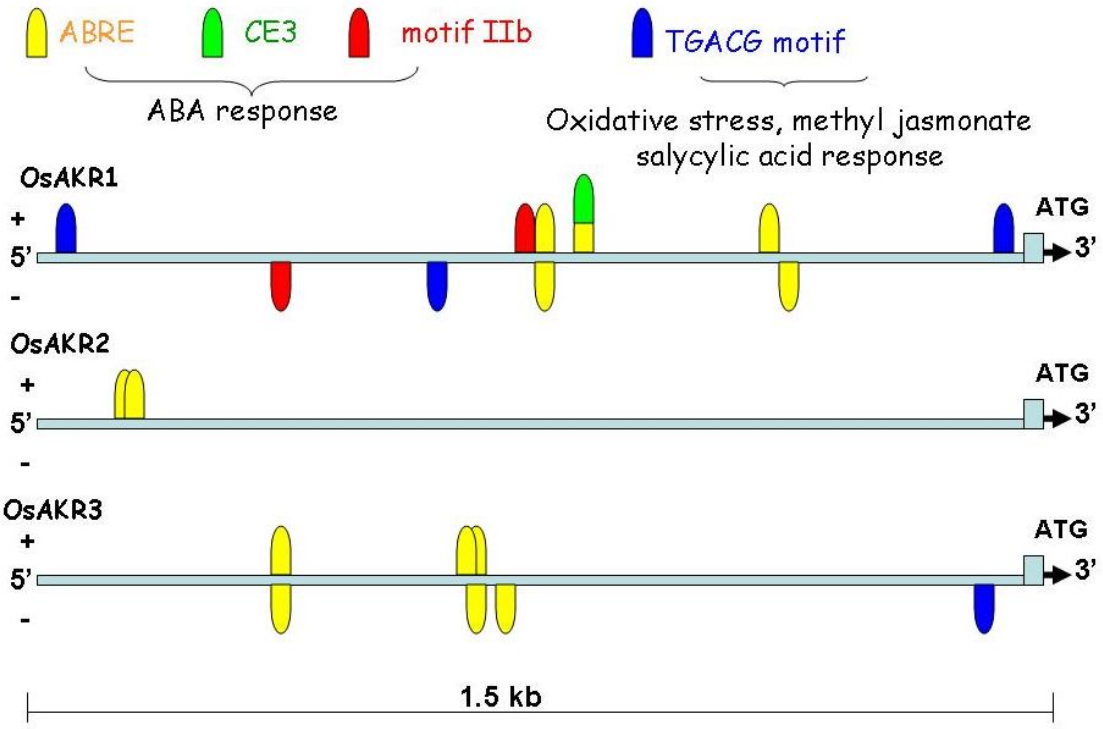


Figure 7. Schematic representation of the potential cis-acting elements involved in ABA and oxidative stress signaling in the promoters of the *OsAKR* genes determined by PlantCARE (a database for Plant Cis-Regulatory DNA elements http://bioinformatics.psb.ugent.be/webtools/plantcare/html). Distances between the promoter elements is approximate. Notice the high number of ABA and oxidative stress related transcriptional factor binding sites in the promoter of the OsAKR1.

Our motif search revealed, that the *OsAKR1* promoter had the highest number of ABREs (ABA-responsive element), CE3 (coupling element associated with ABA response)

and motif IIb (abscisic acid responsive element) among the three rice AKRs, which is positively correlating with the transcriptional response given for ABA treatment. Additionally the *in silico* prediction revealed that *OsAKR1* promoter contained the most TGACG motifs compared to the other two genes investigated. These are binding sites for a variety of transcription factors linked to oxidative stress, auxin, salicylic acid and methyl jasmonate response (Xiang et al. 1996). TGACG motifs are also part of the *as-1* (*activation sequence-1*)-like elements induced by ROS originating from phytohormones or oxidative stress inducers like MV (Garreton et al. 2002). The abundancy of these motifs in the promoter sequence of *OsAKR1* is in good correlation with the high induction profile observed for both ABA and hydrogen-peroxide. In addition, we analyzed the promoters of all the annotated rice AKR genes by using a newly published statistical algorithm, which predicts stress-inducible promoter motifs through the occurrence of putative dyads (Cserháti et al. 2011). From the analyzed genes the *OsAKR1* scored the highest value in the occurrence of stress inducible promoter motifs as compared to the other two candidate genes (*OsAKR2* and *OsAKR3*), once again predicting the high stress inducibility of this AKR.

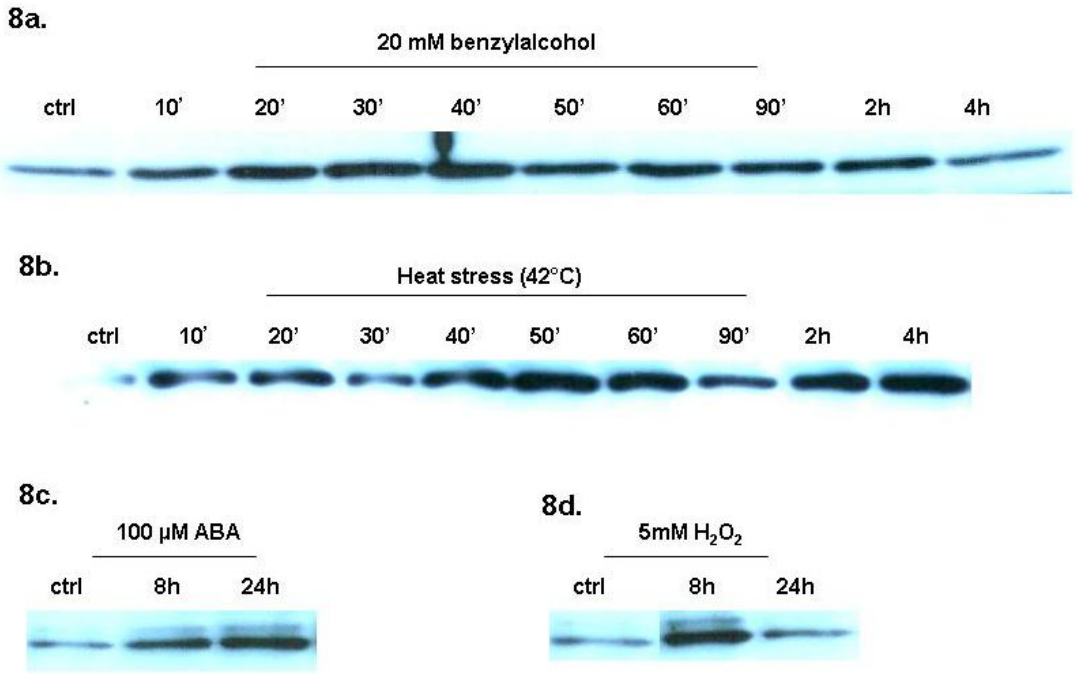
The connection between the promoter elements and the obtained experimental result is debated in detail throughout the discussion. The above presented stress-inducible expression pattern for the *OsAKR1* gene encouraged further studies on the properties of the encoded protein to reveal its role in stress-related reactive aldehyde detoxification pathways.

4.3. Stress agents influencing the AKR protein levels in rice cell suspensions

To complement the transcript analysis we have monitored the changes in the endogenous rice AKR protein levels, as a response to different abiotic stress treatments. The different induction profiles are shown in **Fig. 8a-d**. From the results it is clear, that AKR proteins in rice cell suspensions are induced by a variety of stress factors like heat, benzyl alcohol, abscisic acid and oxidative stress. The antibody raised against the MsALR protein, used for the Western hybridization (described in Materials and Methods), recognizes only a single band in rice cell suspensions which corresponds to AKR, however due to the lack of specificity we cannot distinguish which rice AKR protein species were identified. The patterns of protein induction for ABA and H_2O_2 are in harmony with the transcript profile obtained for

the *OsAKR1* gene (**Fig. 6a**); for both ABA and hydrogen peroxide a late maximum could be observed in the induction pattern at 8 and respectively 24 hrs of treatment (**Fig. 8c, 8d**). However, despite the similar induction profile we cannot state, that the AKR species recognized by the MsALR antibody in rice suspension can be identified as OsAKR1.

Heat stress promotes the accumulation of AKR proteins in rice cell suspensions (**Fig. 8b**) and also the benzyl alcohol (BA), which is a membrane fluidizing agent (Kitagawa & Hirata 1992) that mimicks the effect of elevated ambient temperatures.



Figures 8a, 8b, 8c, 8d. Accumulation of rice AKR protein species in cell suspensions following different abiotic stress treatments. The α-MsALR antibody and the peroxidase (POD) anti-rabbit IgG antiserum was used for the immunodetection. Same amount of protein (10 µg) was loaded in each lane.

The effect of BA treatment on the rice cell suspensions can be described with an early AKR protein induction, reaching the peak after 40 min and then a gradually decrease in later timepoints (**Fig. 8a**). Heat stress is causing a slightly different induction profile, with one early maximum at 50 min and then a late peak after 4 hrs (**Fig. 8b**). These results suggest, that

certain rice AKR species might play important role in the adaptive metabolic response following high temperature associated damages, possibly through their envolvement in the follow up protection, through reactive carbonyl scavenging.

4.4. Enzyme kinetic parameters of OsAKR1

Based on the promoter analysis and the gene expression data we have selected the *OsAKR1* gene as the top candidate for detailed functional characterization. As a first step, we were curious about the kinetic properties of this enzyme. Glutathione-Sepharose 4B column purified recombinant GST-OsAKR1 protein (3 μg for one measurement) was used for the biochemical characterization. The purified GST-OsAKR1 (**Fig. 9**) was found to be active in the presence of various aldehydes and also with one sugar substrate (D-xylose) when using NADPH as a cofactor, but was inactive with all the above substrates with NADH.

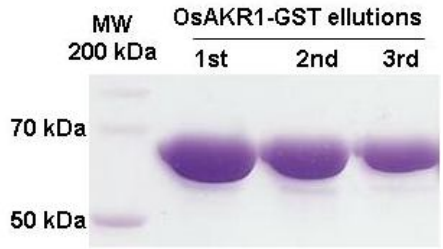


Figure 9. Purified OsAKR1-GST recombinant protein (approx. 64 kDa) following elution with 10mM reduced gluthatione. Same volume of the eluted fractions was loaded on the SDS poliacrylamide gel.

The use of GST-OsAKR1 fusion protein for the enzyme kinetic measurements was consolidated by our previous studies on thrombin-cleaved MsALR (Oberschall et al. 2000) showing that the GST fusion partner did not affect the enzyme activity of the AKR protein. Substrate specificity of this aldo-keto reductase is shown in **Table 1**.

Substrate	*Constant*	*OsAKR1 values*	*MsALR values*	*hGR values*
NADPH	Km (μM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	37.9 ± 4.4 4.8 ± 0.4 $1.3\cdot 10^5 \pm 2.7\cdot 10^4$	4.3 2.2 $5\cdot 10^4$	2 1.1 $5.5\cdot 10^5$
DL-glyceraldehyde	Km (mM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	2.1 ± 1 4.8 ± 0.7 $3\cdot 10^3 \pm 1.5\cdot 10^3$	3.2 6 $1.8\cdot 10^3$	3.4 9.3 $2.8\cdot 10^3$
Malondialdehyde	Km (mM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	0.5 ± 0.1 0.26 ± 0.03 550 ± 83	ND ND ND	- - -
Methylglyoxal	Km (mM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	0.4 ± 0.18 6.3 ± 1.1 $1.8\cdot 10^4 \pm 4.8\cdot 10^3$	1.55 16.8 $1.1\cdot 10^4$	1.2 3.57 $3\cdot 10^3$
Glyoxal	Km (mM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	7.6 ± 0.15 4.4 ± 1.9 578 ± 250	8.3 * 6.4 768	- - -
Phenylglyoxal	Km (mM) kcat (sec^{-1}) kcat/Km ($sec^{-1}M^{-1}$)	0.07 ± 0.02 5.7 ± 2 $8.8\cdot 10^4 \pm 3.8\cdot 10^3$	0.042 * 4.3 $1.4\cdot 10^4$	- - -

D-xylose	Km (mM)	120.3 ± 45.8	ND	-
	kcat (sec^{-1})	4.7 ± 0.8	ND	-
	kcat/Km ($sec^{-1}M^{-1}$)	41.5 ± 10.1	ND	-

Table 1. Apparent kinetic constants of the recombinant OsAKR1 in comparison with the MsALR (Oberschall *et al.*, 2000) and the human aldehyde reductase (HuGR) (Bohren *et al.*, 1991, Van der Jagt *et al.*, 1992). OsAKR1 values are the average of at least 3 measurements.

*MsALR constants for phenylglyoxal and glyoxal, determined by us during the course of this study

Among the substrates of OsAKR1, MDA, the major lipid peroxidation product can be considered of great importance. MDA is generated in plant cells during oxidative stress and it is important to emphasize, that the OsAKR1 enzyme showed considerable activity on this substrate with a Km value much closer to its predicted *in vivo* concentration than it was reported for an *A. thaliana* AKR encoded by the *At2g37770* gene (Simpson et al. 2009). This activity of the aldo-keto reductases on MDA substrate is not recorded in The Comprehensive Enzyme Information System (BRENDA, http://www.brenda-enzymes.org/), although Halder et al. (1984) gave similar kinetic parameters for an AKR purified from bovine lens to those we obtained with GST-OsAKR1 protein. Based on both Km and kcat/Km values, this rice enzyme shares similarity with the recombinant MsALR (Oberschall et al. 2000) and the human aldehyde reductase (Bohren et al. 1991; Vander Jagt et al. 1992). We conclude that the OsAKR1 has higher affinity to aldehyde and oxoaldehyde substrates (methylglyoxal, phenylglyoxal) than to D-xylose, which was only poorly accepted as a substrate. Therefore we suggest that the OsAKR1 enzyme should be considered as an aldehyde reductase rather than an aldose.

Although *OsAKR1* was the primary focus, as being the most stress inducible and containing the highest number of putative abiotic stress related transcriptional factor binding sites in the promoter, we were still curious to check the substrate specificity the other two recombinant proteins, coded by *OsAKR2* and *OsAKR3* genes. We have made several attempts in bacterial systems using different epitope tags; moreover we also used a cell-free wheat germ extract based *in vitro* translation system. Although proteins could be purified, we were unable to get significant activity for the substrates we tested for MsALR and OsAKR1. There might

be several reasons behind this observation; it could be the differences in their predicted secondary structure, inactive form of purification in either prokaryotic or eukaryotic systems, or such a narrow substrate specificity which we could not identify (see Discussion).

4.5. *In vitro* modification of a Calvin cycle enzyme by RCOs

A variety of lipid peroxidation and/or glycolysis derived aldehydes can modify proteins. The term protein carbonylation (see Introduction), refers to the attachment of carbonyl residue on amino acid side chains of proteins (cystein, lysine). It is a covalent modification which usually results in the loss of protein activity. OsAKR1 accepts both MDA and MG as substrates as shown by the enzyme kinetic parameters. The experimental proof that indeed MDA and MG are able to modify and thereby to inactivate functional proteins was also our concern. In order to show exactly how a biologically significant, toxic aldehyde is able to modify proteins, we have performed inhibition assays on an important enzyme of the photosynthetic Calvin cycle, the PRK. We have cloned the *Arabidopsis thaliana PRK* gene (identifier *At1g32060*) into pET-28a expression vector and we have purified recombinant PRK-His protein from bacterial system. The Ni-affinity column purified PRK is shown in **Fig. 10**.

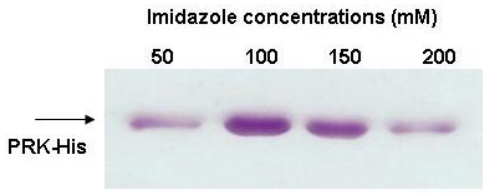


Figure 10. **Purification of recombinant phosphoribulokinase from *E.coli* BL21-Gold strain, following ellution by imidazole from Ni-affinity column**. Equal volumes are loaded on the SDS-PAGE gel

Being an enzyme regulated by the reversible oxidation/reduction of sulfhydryl and disulphide groups it is a plausible target for reactive aldehyde modification in the chloroplasts, due to the presence of cysteins in the active domain. Incubation of PRK with 1 mM MDA resulted in the apparition of high molecular weight bands on the membrane hybridized with the R-α-DNP antibody (**Fig. 11a**). MDA treatment led to a gradual loss of the PRK activity (**Fig. 11d**). The inactivation profile was similar when PRK was treated with 5mM MG; after 1 and 2 hrs of

incubation, most of the enzyme activity was lost (**Fig. 11e**). Via the immunochemical detection, protein oxidation pattern induced by MG was different then for MDA (**Fig. 11b, Fig. 11a**). In the case of MG the immunodetection was performed as the aftermath of a previous experiment, which showed, that incubation of PRK with high concentrations of MG (1 mM, 5 mM, 10 mM, 30 mM and 100 mM) results in the apparence of high molecular weight bands on the Coomassie stained SDS-polyacrilamide gel (**Fig. 11c**). The intensity of these bands increased along with the concentration and resulted in the dissapearence of the band corresponding to the unmodified recombinant PRK. These high molecular weight bands could be attributed probably to the protein-crosslinking effect of the MG, if present in high concentrations.

Fig 11a **Fig 11b**

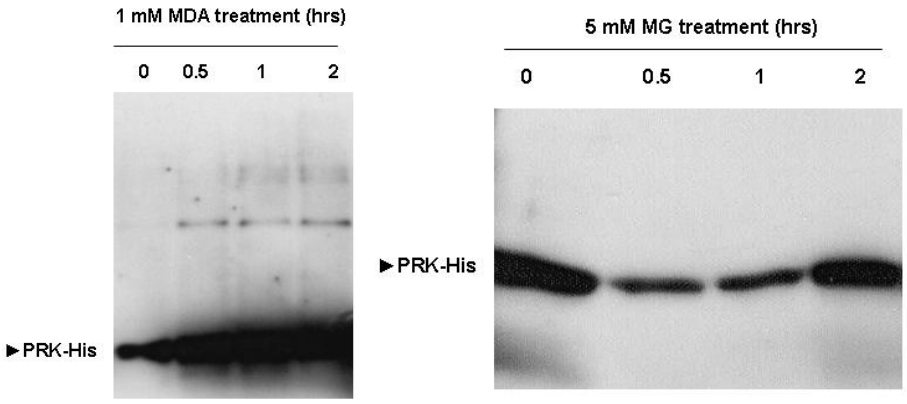


Fig 11c

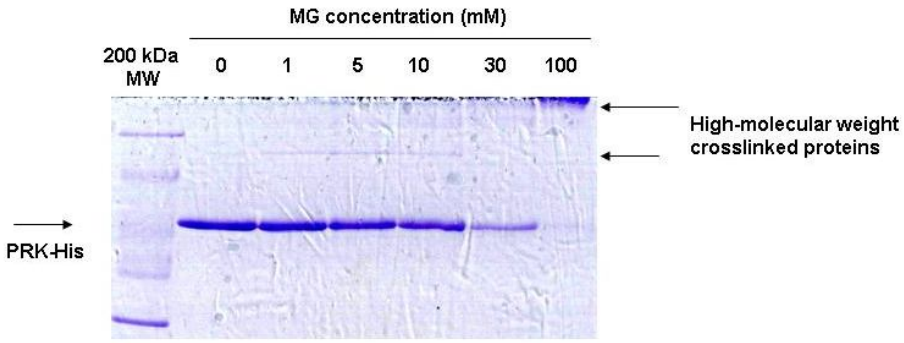


Figure 11a, b. **Immunochemical detection of protein oxidation in MDA and MG treated PRK**. Notice the presence of additional high molecular weight protein bands and the increased level of carbonylated product with treatment time on the membrane containing samples modified with MDA and MG. **Figure 11c. PRK**

modification with high concentrations of MG. Samples were treated with MG for 10 hrs on 37ºC. Notice the correlation between the concentration increase and the dissaperance of the protein band corresponding to native, unmodified PRK being substituted by the crosslinked products. Equal amount of protein was used in all cases.

Fig 11d

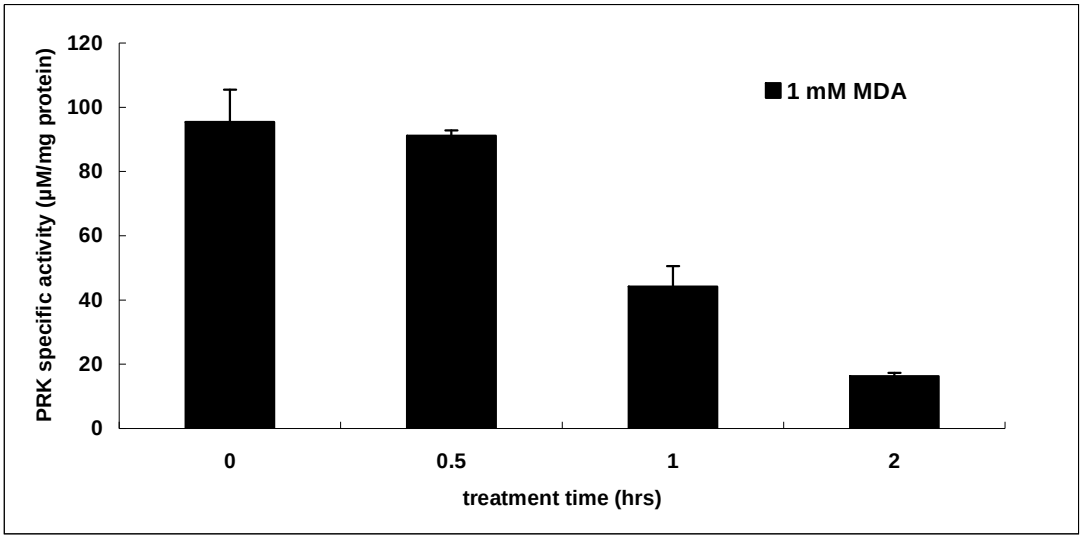


Fig 11e

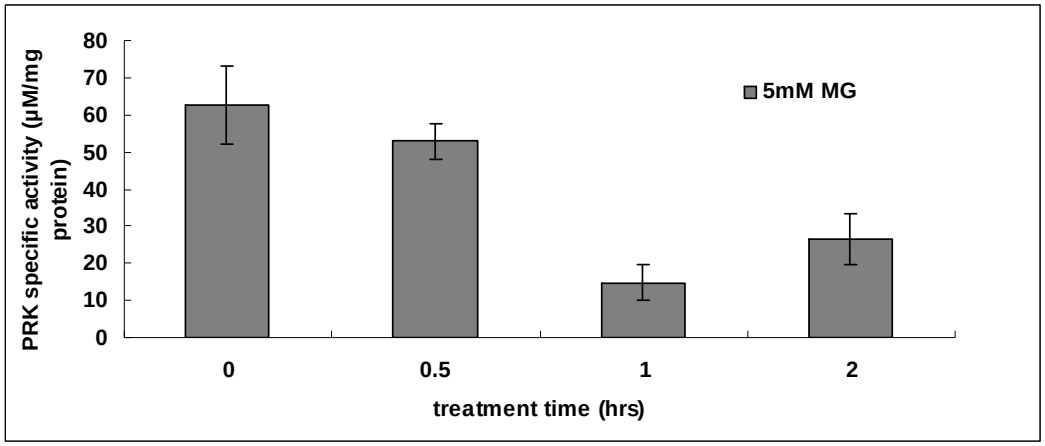


Figure 11d, e. Inhibition of PRK activity by 5mM MG and 1mM MDA. Bars represent the specific activity in correlation with the treatment time. Results are the average of 3 independent measurements ± St. Dev

The interaction of PRK with both MG and MDA inhibited the enzymatic activity and caused covalent modifications on the protein, such as crosslinking and carbonylation (**Fig. 11a-e**).

These examples show how important it is to effectively detoxify reactive carbonyls before they accumulate in excess amounts in order to prevent the modification and inactivation of biologically important proteins.

4.6. Tolerance of OsAKR1 overproducing *E.coli* cells to MG

This experiment was performed in order to get a glimpse at the *in vivo* effect of OsAKR1 overexpression in a phylogenetically distant organism (bacteria) against the toxic carbonyl compound, MG. *E.coli* cells containing the *pGEX4T-1::OsAKR1* construct and expressing the rice AKR fusion protein (GST-OsAKR1) were grown in standard LB media and 2 mM MG solution was exogenously applied to the cultures. Presence of the GST-OsAKR1 recombinant protein in the bacterial cells was quantified by Western blot. The survival rate of the bacterial cells determined from treated vs. control ratios was significantly higher in cells overproducing the GST-OsAKR1 fusion protein, than in cells with GST only (**Fig. 12**).

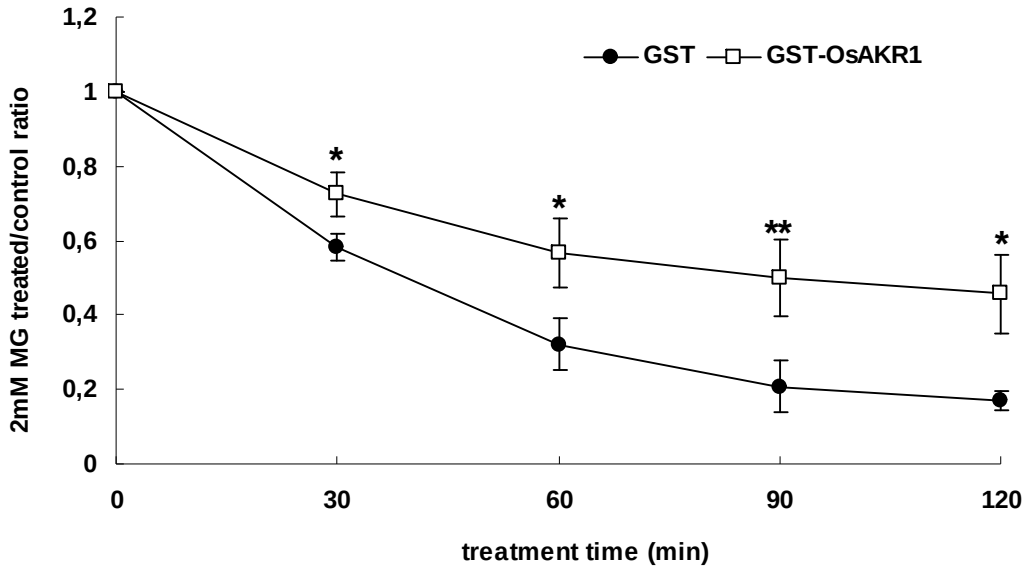


Figure 12. *In vivo* protective effect of GST-OsAKR1 protein production against MG toxicity in *E. coli*. BL21 (DE3) cells containing either the control plasmid pGEX-4T1 or the OsAKR1 expression plasmids were grown in LB to the early exponential phase before the addition of IPTG for 60 min to induce the expression of GST alone or GST-OsAKR1 fusion protein. Cells were treated with 2mM concentration of MG for 2 hrs, the number of viable cells was determined at every 30 minutes. Values represent the mean ± S.E.M. The asterisks indicate significant differences at $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisk) respectively.

Bacterial cells with high levels of *OsAKR1* were more resistant to MG exposure than the control cells. This finding supports an *in vivo* protective function for OsAKR1 against MG toxicity in bacteria. Similarly the results of Grant et al. (2003) show, that *E.coli* cells overexpressing an AKR protein identified from the same bacteria (YghZ) belonging to the AKR14A1 family led to higher tolerance to increasing concentrations of MG then the control. In cyanobacteria (*Synechococcus* PCC 7002) an AKR null-mutant strain accumulated more toxic MG than the wild type and was unable to efficiently detoxify exogenous MG compared to the non-mutant strain (Xu et al. 2006). Our result is important in the demonstration of the conserved function for these proteins; a rice AKR protein is able to detoxify MG in a bacterial organism by complementing the function of the endogenous proteins.

4.7. Transgenic tobacco lines overexpressing OsAKR1

Through *Agrobacterium* mediated transformation we obtained 17 independent transgenic tobacco lines, heteorogously overexpressing the *OsAKR1* cDNA. Positive lines were confirmed through PCR and Western blotting, with the antibody raised against MsALR. Three lines from the T3 generation were selected for further experiments, based on the accumulating OsAKR1 protein (**Fig. 13**).

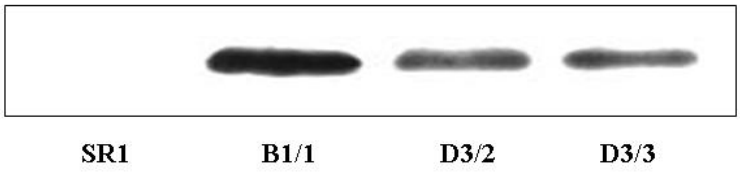


Figure 13. Western hybridization showing the OsAKR1 protein levels in the transgenic lines used for the stress experiments. Equal amount of protein (10μg) was loaded on the SDS-PAGE gel. No protein in the first lane represents the wild type plant.

As we can see from the Western hybridization data, the B1/1 line accumulated the highest amount of OsAKR1 protein among the transgenic lines. The use of anti-MsALR antibody to screen the genetic transformants, to identify the positive tobacco lines and at the same time to determine the accumulated OsAKR1 protein level was consolidated by the fact, that this antibody does not hybridize with endogenous tobacco AKR proteins (no band was detected in the wild type SR1).

4.8. OsAKR1 overexpression increases the tolerance against toxic concentrations of MG in plant system

Previously we have seen the effect of OsAKR1 overproduction in *E.coli*; the viability of the overexpressing cells was much higher than in the controls upon exposing the cells to 2 mM MG. As a sequel to this experiment we could show, that the overexpression of this transgene in tobacco plants resulted in extended survival upon excessive 10 mM MG treatment as well, reflected by the photosynthetic parameters (**Fig. 14**).

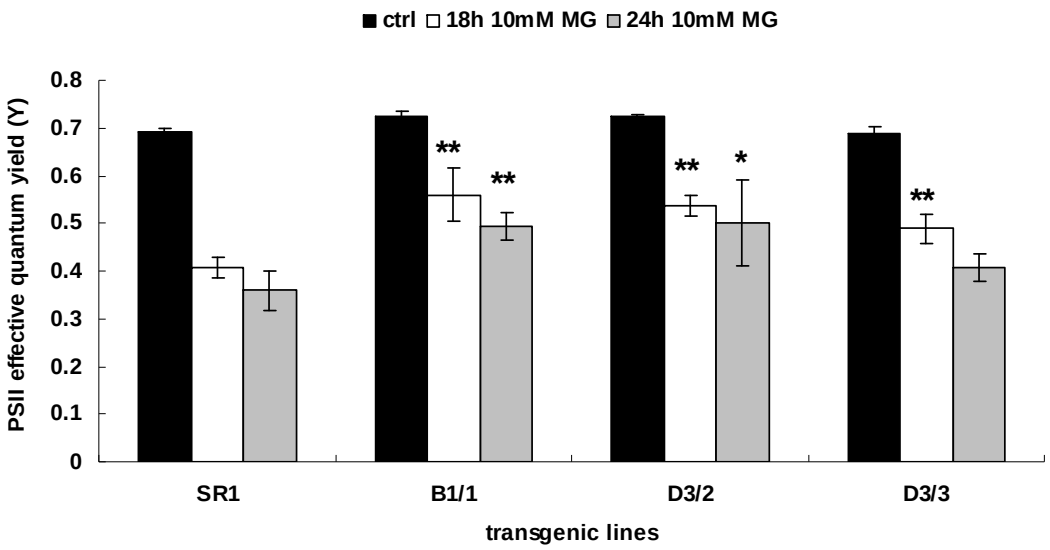


Figure 14. **PSII effective quantum yield of OsAKR1 over-expressing tobacco plants treated with 10 mM methylglyoxal.** SR1 represents the wild type control. Significant differences from SR1 are at $P < 0.01$ (two asterisks) and $P < 0.05$ (one asterisks).

After 18 hrs of strong cytotoxic effect the PSII effective quantum yield in the SR1 plants dropped to approximately half of the control values, whereas in the transgenic lines (especially B1/1) only a subtle decrease could be observed compared to the non-treated values. Within 24 hrs of treatment both the B1/1 and D3/3 transgenic plants still maintained a significantly higher photosynthetic rate then SR1 (**Fig. 14**). Here also we can establish a correlation between the expressed OsAKR1 protein level by the transgenic tobaccos and the degree of tolerance; B1/1 line showed the highest expression of the foreign protein and consequently presented higher tolerance to MG treatment. These data support the hypothesis that the overproduction of OsAKR1 is beneficial in reactive carbonyl scavenging.

4.9. Decreased reactive carbonyl content in transgenic tobaccos under abiotic stress conditions

Unfavorable conditions often accelerate the rate of certain pathways leading to excessive accumulation of reactive compounds. Such are MDA, originating mainly from linolenic acid derived peroxidation and MG, an important glycolysis by-product. We have

compared the level of these reactive carbonyls between transgenic and wild type plants under control, and heat stress conditions. High temperature stress was applied through a 50 min treatment in 44 °C water bath (see Materials and Methods). Following heat exposure, the leaf discs were placed in distilled water and left to recover for 48 hrs at room temperature, before they were assayed for MDA and MG content. The 48 hour recovery period was implemented based on the observations of Larkindale and Knight (2002) on heat stressed *Arabidopsis* plants, where they showed massive increase in TBARS following 2 and 3 days of recovery, but no increase after 1 day. This is consistent with our observations. In the case of MDA, there was no significant difference in the control stage, however following heat stress higher MDA levels could be detected in the SR1, whereas significantly lower in the transgenic plants compared to the controls (**Fig. 15a**).

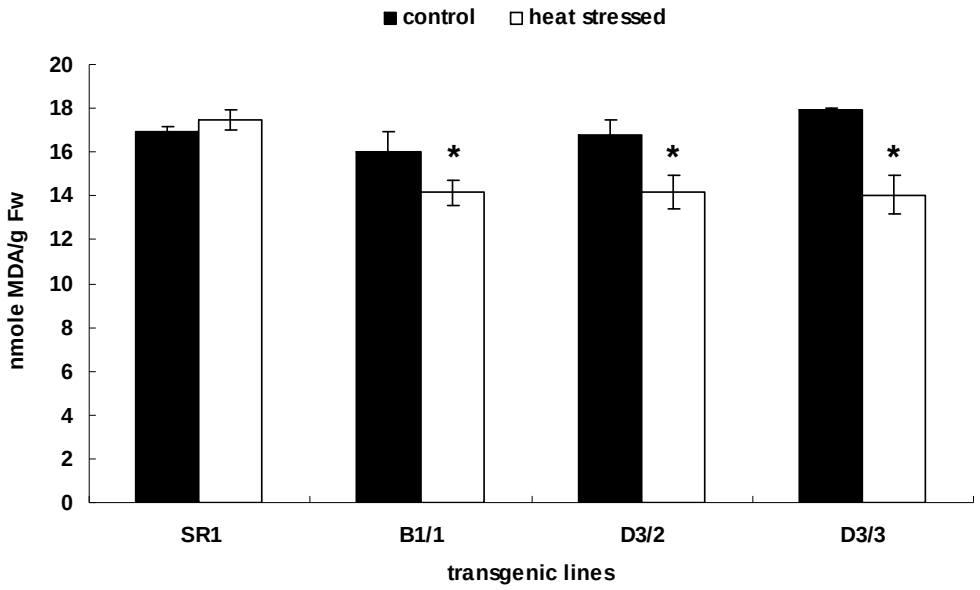


Figure 15a. MDA levels in wild type and transgenic plants before and after heat exposure. The applied heat treatment was 44 °C for 50 minutes, and following treatment, the leaves were transferred in distilled water and kept there for 48 hrs, under constant light intensity. The bars represent the results of 3 independent experiments ± SD. Significant differences from SR1 are represented by one asterisk ($P < 0.05$).

Heat treatment did not cause a considerable increase in the MG level; nevertheless we could see lower levels of MG in the transgenic lines compared to wild type both in the control stage and following heat stress (**Fig. 15b**). It seems that the overproduction of this enzyme prevents the accumulation of MG in the transgenic plants, even without stress conditions, as reflected

by the reduced amounts in the overexpressing lines compared to the SR1 already in the control stage.

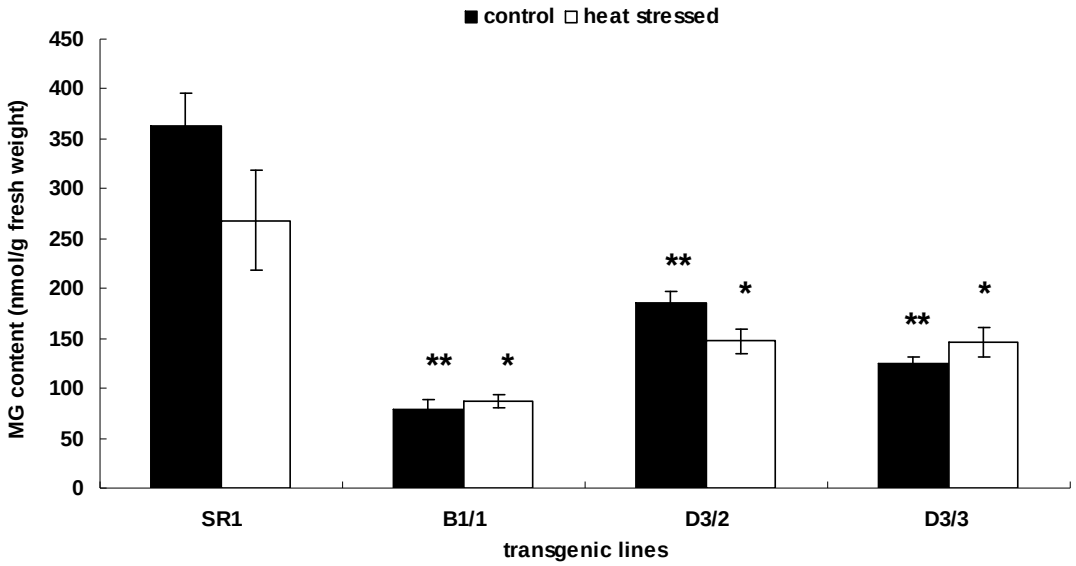


Figure.15b. **Significantly lower methylglyoxal levels measured in the transgenic lines compared to wild type both before and after heat stress.** The amount of MG was measured spectrophotometrically, using standard methylglyoxal solutions. The values represent the average of at least 3 measurements ± SD. Significant differences from SR1 are at $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisk) respectively.

Two conclusions can be derived from these data: Firstly, heat stress caused a slight increase of MDA in the wild type plants, but not in the transgenic ones. The MDA levels in the transgenic lines were lower after heat stress then in the control, suggesting a more effective detoxification, presumably due to the constitutive overexpression of OsAKR1. Secondly, the overproduction of OsAKR1 may be helpful in regulating the endogenous level of certain reactive carbonyls (e.g. MG) by complementing the effect of other detoxifying pathways (e.g glyoxalase pathway). The amount of detoxifying enzyme available prior to the stress conditions might be important in the effectiveness of the overall scavenging mechanism. It is worth noticing, that the B1/1 line expressing high amounts of OsAKR1 protein (**Fig. 13**) had a notable difference from the other two verified lines in terms of MG content following heat treatment. MDA levels were generally lower in all the transgenic lines following heat stress

than in SR1 and in this case there was no significant difference among the transgenic lines. Therefore it seems obvious that reduced amounts of these aldehydes are the consequence of overexpression, since both reactive carbonyls are actively metabolized by OsAKR1 *in vitro* as well.

4.10. Oxidative and heat stress tolerance of the OsAKR1-overexpressing tobacco plants

MV treatment generates ROS (notably superoxide anion radicals) (Kwon et al. 2002), therefore increasing the rate of lipid peroxidation and the accumulation of MDA (Rodrigues et al. 2006). We measured this product as TBARS in MV treated leaf discs obtained from three independent transgenic lines (D3/3; D3/2; B1/1) and the wild type SR1. Shown by **Fig. 13** the transformed tobacco plants synthesized significant amounts of rice OsAKR1 protein. As a consequence, the amount of TBARS in the transgenic lines did not change significantly even after the 2 μM MV treatment while already 1 μM MV caused a considerable increase of the TBARS level in the SR1 plants (**Fig. 16**). The biggest difference between the wild type plants and transgenic lines we can repeatedly notice for the B1/1, as being the highest OsAKR1-expressing line.

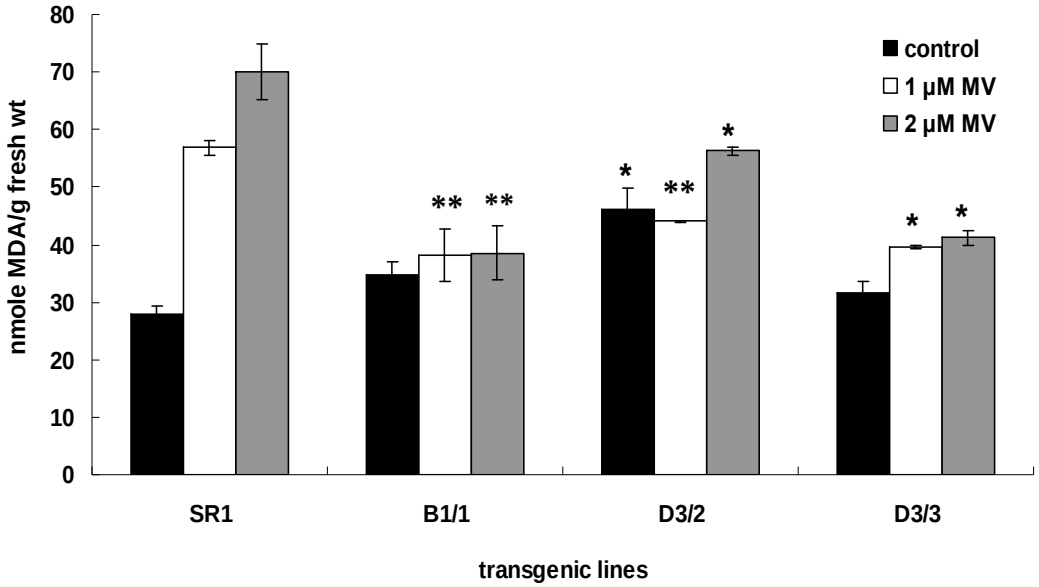


Figure 16. Overproduction of OsAKR1 in transgenic tobacco significantly reduced the methylviologen-induced reactive carbonyl accumulation. Values are represented as mean ± StDev. The bars indicate the MDA concentration. The asterisks indicate significant differences from SR1 at $P < 0.01$ (two asterisks) and $P < 0.05$ (one asterisks)

A reduced accumulation of lipid peroxidation-derived aldehydes in the transgenic lines supports our hypothesis that over-expression of *OsAKR1* may enhance the detoxification process. When whole, transgenic and wild type tobacco plants were subjected to 100 µM MV treatment, visible morphological changes could be observed on the leaves (chlorosis, bleaching). This is likely due to the rapid accumulation of ROS and ROS-induced secondary products which cause cellular injury and the impairment of the photosynthetic process by MV. **Fig. 17a** clearly shows that the morphology of the *OsAKR1* overexpressing lines was visibly better (less chlorotic leaf spots) under oxidative stress as compared to the wild type under high concentration of the applied herbicide (100 µM MV). This suggests a more efficient detoxification of the reactive compounds generated through the MV treatment in the transgenic lines, supported by significantly lower MDA levels detected in the B1/1, D3/2 and D3/3 plants following 48 hrs of treatment compared to the wild type (**Fig. 17b**). Among the transgenic lines the B1/1 line had the most significant difference from the SR1, probably due to the high amount of expressed OsAKR1.

Fig 17a

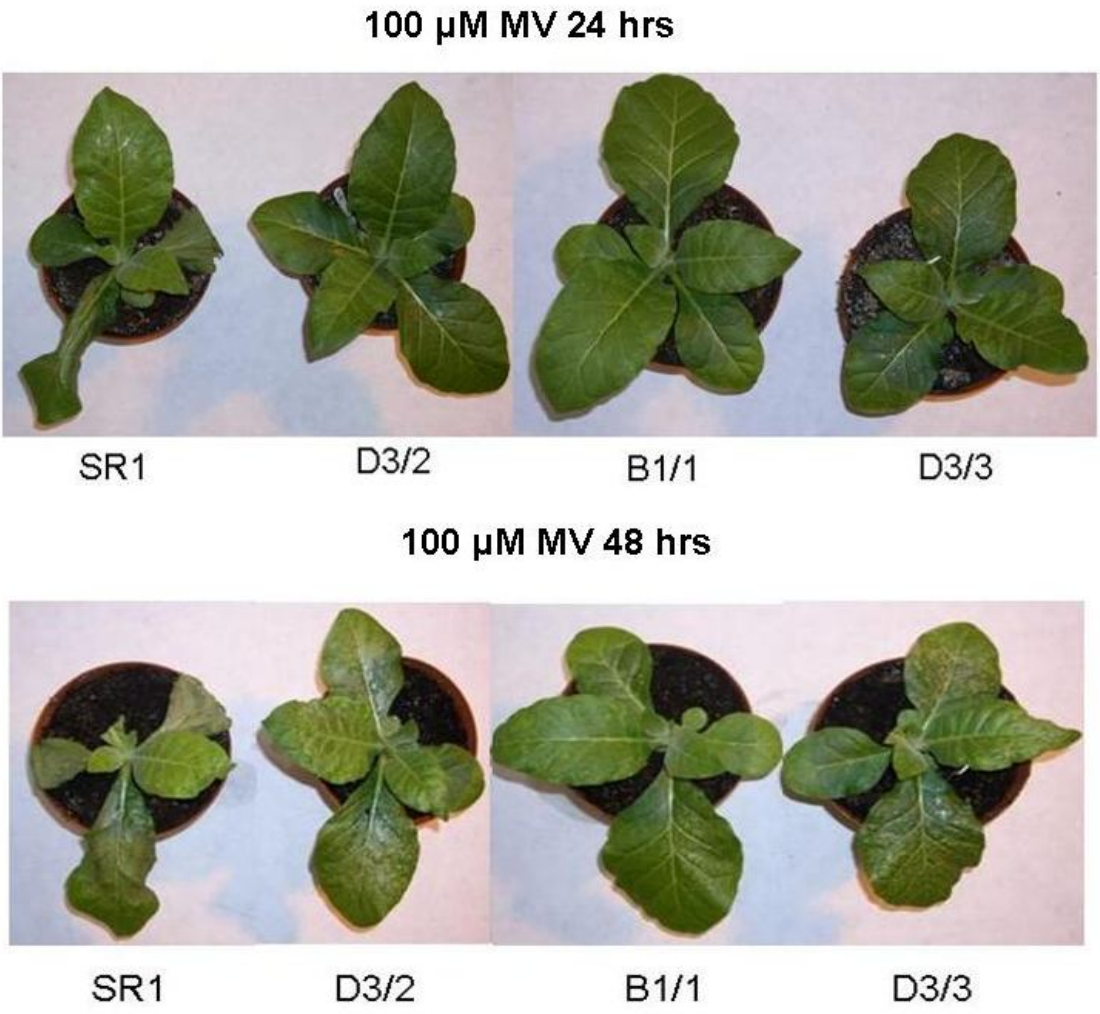


Fig 17b

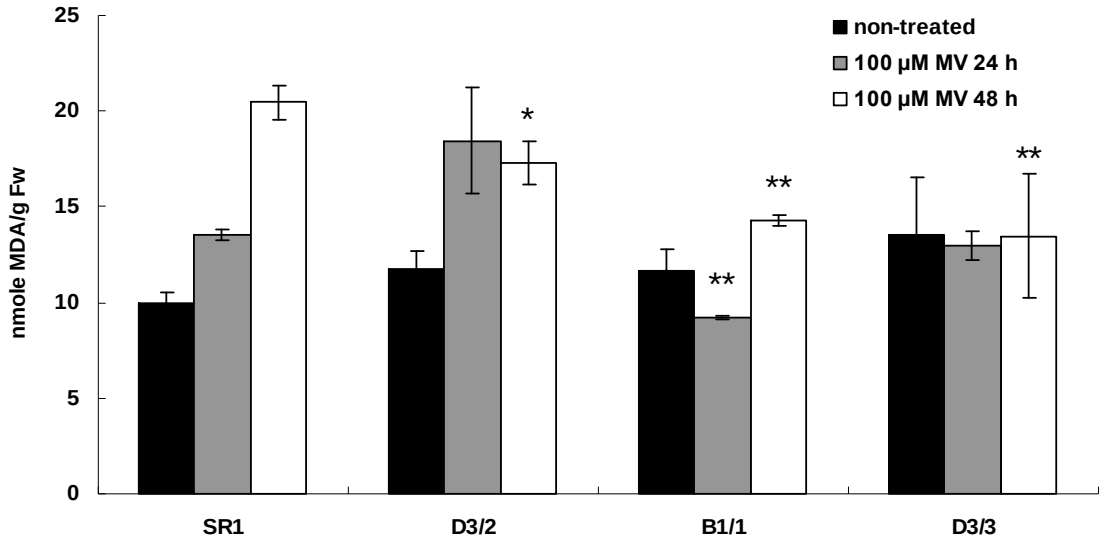


Figure 17a, b. Effects of oxidative stress on the transgenic plants overexpressing OsAKR1 compared to the wild type.a) Photographs showing visible differences in the condition of 6 week old transgenic and wild type tobacco plants that were sprayed with 100 µM of MV and grown in the greenhouse under the same light conditions as described in *3.4* b) Lower MDA levels in the transgenic plants compared to the SR1 sprayed with 100 µM MV after 24 and 48 hrs of oxidative damage. Significant differences from SR1 are at $P < 0.01$ (two asterisks) and $P < 0.05$ (one asterisks).

The three transgenic lines showing higher tolerance to oxidative stress generated by MV (**Fig. 17a, b**) proved to be more tolerant to high temperature stress as well. This is demonstrated by a significant difference between photochemical yields of *OsAKR1* transformants and the wild type SR1 after 50 minutes at 44 °C (**Fig. 18**).

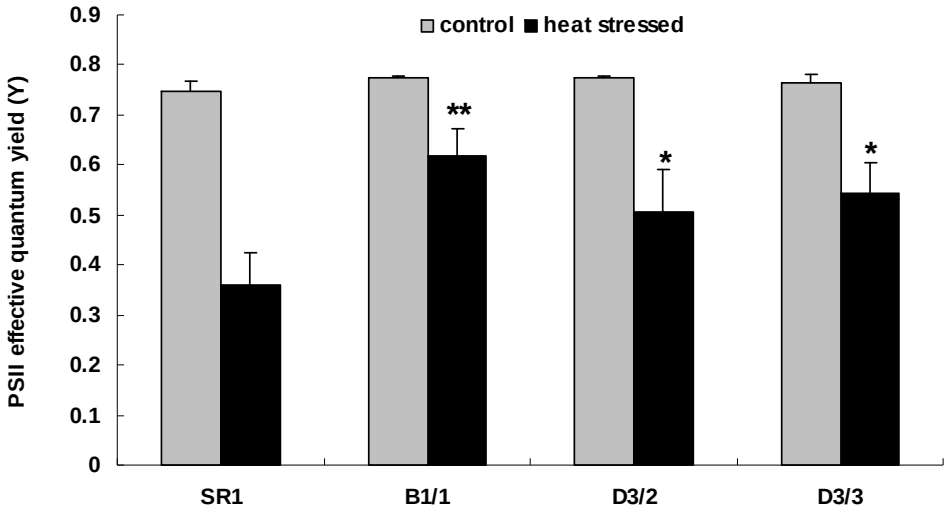


Figure 18. Improved tolerance following heat stress reflected by the photosynthetic parameters of the OsAKR1 overproducing transgenic lines (B1/1, D3/2, D3/3) compared to wild type (SR1) as reflected by the photochemical yield. The bars indicate the measured PSII effective quantum yield of the control plants (*grey* bars) and the heat treated plants (*black* bars). Data represents the average of 3 measurements ± SD. The asterisks indicate significant differences from SR1 at $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisk) respectively.

The overexpressing B1/1 line had the highest photosynthetic yield after the heat treatment, in accordance with having the highest OsAKR1 protein content (See **Fig. 13**). Additionally, the other two transgenic lines were also more resistant to the high temperature treatment than SR1, but to a smaller extent than B1/1. Their differences in photochemical yields could be correlated with the amounts of OsAKR1 protein expressed by the transgenic lines, as the other two lines produced less rice AKR protein then B1/1. This was further demonstrated by comparing the AKR activity of the leaf extracts originating from the transgenic lines vs. wild type before and after heat stress. Line B1/1 had higher AKR activity compared to the SR1 and so did the other leaf extracts (although more moderate) from line D3/2 and D3/3 (**Fig. 19**).

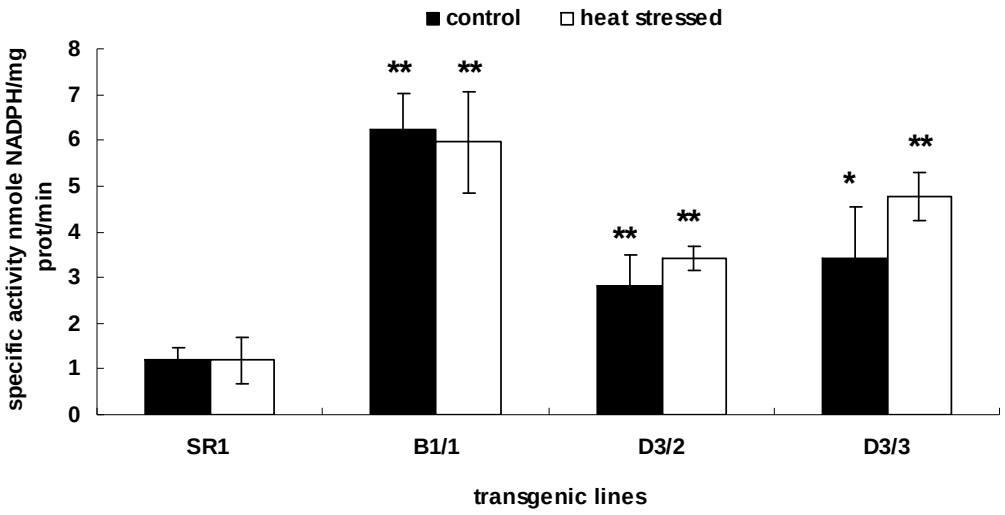


Figure 19. Higher AKR activity in the transgenic lines before and after heat stress, compared to the wild type SR1. Total aldo/keto reductase activity was measured on leaf extracts, with methyglyoxal as a substrate. The data represent the average of three replicates, and significant differences can be seen at $P < 0.05$ (one asterisks) and $P< 0.01$ (two asterisks)

There was hardly any difference in the activity measured before and after heat treatment in all the lines, suggesting that the amount of scavenging enzyme (OsAKR1) available prior to the initiation of stress stimuli might be partially responsible for the coordination of heat stress tolerance. These data indicate a possible correlation between heat stress tolerance and the amount of accumulated OsAKR1 protein in the transgenic plants (Turóczy et al. 2011).

5. DISCUSSION

5. 1. Classification of rice AKR genes

Sequencing of the rice genome and the availability of large EST (expressed sequence tag) databases enables relatively easy genome wide search for genes involved in stress responses. Our work started with the aim to identify and classify all the annotated rice AKR genes and to select candidates for detailed characterization based on their homology with other previously characterized stress responsive counterparts from different plant species. Other criteria was the mapping of stress related QTLs in the chromosomal regions where the AKR genes are located. The sequencing of the chr1 was finished and published in 2002 (Sasaki et al. 2002). Since the annotation of genes on *Chr1* was considerably accurate, we began to look for chromosomal regions on this particular chromosome, containing putative stress-inducible AKR genes. After extensive *in silico* searches, our attention focused to a chromosomal region containing three highly homologous AKR genes in the neighbourhood of the well-known, stress inducible proline biosynthesis (*P5CS2*) gene. The hypothesis that this chromosomal region might have role in the abiotic stress adaptation of rice was supported by the identification of mapped drought- and other abiotic stress-related QTL's for this chromosomal region, using the rice QTL Genome Viewer, from the National Institute of Agrobiological Sciences QTL Genomics Research Center webpage (http://www.nias.affrc.go.jp/qtl/english/index.html).

The candidate rice AKR genes located in this region showed considerable homology with the previously characterized stress responsive *MsALR* (Oberschall et al. 2000) and other *AKR* genes from different species (e.g. the recently characterized *At2g37770* (Simpson et al. 2009). Previously we have also scanned the *Arabidopsis* genome for stress inducible *AKR* genes based on the transcriptomic information obtained from the Geneinvestigator database (data not shown) and we have found several stress responsive *AKRs* (e.g. *At2g37770*) that have high homology with our rice genes. It is worth mentioning that according to the phylogenetic tree (**Fig. 5a**) containing all the putative rice *AKR* genes until now, some genes (particularly the *Os05g0456300*) show higher homology to both the *MsALR* and *DpARs* then *OsAKR1, OsAKR2* or *OsAKR3*. Still these genes did not become the focus of our research due to the reason that *Chr5* genes were not annotated at that time.

Till now, by performing a genome wide search, we have found 25 annotated rice *AKR* genes. We have aligned these genes to some of the previously characterized stress responsive plant *AKRs*, like *MsALR* (Obershall et al. 2000), *DpAR1 and DpAR2* (Gavidia et al. 2002), *ALRXV4* (Mundree et al. 2000), *HvALR* (Roncarati et al. 1995), *At2g37770* (Simpson et al. 2009) Following alignment of the three rice protein sequences (OsAKR1, OsAKR2 and OsAKR3) with representative AKRs from the superfamily, and analysis of percentage identities as well as their functional characteristics they were assigned into the AKR4C subfamily of aldo-keto reductases.

5. 2. OsAKR1: an abiotic stress-inducible gene

For filtering the rice genome in search for stress-responsive AKRs, (particularly the *Chr.1*) we applied different *in silico* methods; determining chromosomal localization, mapping of stress associated QTL's, homology searches, promoter analysis etc. As a result, we narrowed down our interest to three highly homologous *AKR* genes situated in a short chromosomal region where numerous, mainly drought and other abiotic stress-related QTL's have been mapped. This might be due to the location of the *P5CS2* gene in this chromosomal region, a gene well known for its role in the abiotic stress responses. These three *AKR* genes are highly homologous to each other and we hypothesize that they might be the result of a gene duplication event, which might have occurred in the rice genome. We verified the transcriptional response of the newly identified *AKR* genes in response to different stress treatments and found that the gene denoted by us as *OsAKR1* showed the highest transcriptional response, induced by a wide range of factors including ABA and hydrogen-peroxide as the most effective inducers. The other two genes (*OsAKR2* and *OsAKR3*) located in the same chromosomal region were moderately stress inducible.

Aldose reductase homologues have already been characterized in monocot species. In barley, the corresponding gene was expressed exclusively in embryos and influenced by ABA (Bartels et al. 1991). In rice, an aldose reductase-related protein was also detected in mature seeds, and shown to acculmulate in vegetative tissues following dehydration, salt-stress and exogenous ABA treatment (Sree et al. 2000). These data are in good agreement with the increased gene transcription observed in the present study for the *OsAKR1* gene responding to

ABA treatment and oxidative stress. The other two genes (*OsAKR2, OsAKR3*) were moderately ABA inducible. The differences in the transcriptional induction can be attributed to the quantity and quality of the cis-acting regulatory elements present in the promoters (**Fig 7**).

The number of *in silico* predicted stress inducible elements in the promoter and the actual stress inducibility of a gene might be different, still often there is a correlation between these two. It was suggested that most ABA-responsive genes have conserved ABRE elements in their promoters, which are significant elements for genes responsive to abiotic stress (Mundy et al. 1990, Xu et al. 1996). In our case the presence of a high number of ABRE elements in the promoter of *OsAKR1* analyzed by PlantCARE was experimentally supported. Since these promoter elements often have a well-defined core motif, the location of these regulatory cis-elements can be determined in the sequence, taking in consideration other aspects such as, the gap lengths between the motifs, distance from the translational initiation codon etc. ABRE contains an ACGT core, a sequence known to be recognized by plant basic leucine zipper (bZIP) proteins. It has been established that a single copy of ABRE is not enough for ABA mediated induction of transcription, but multiple ABREs or the combination of an ABRE with a coupling element (CE3) motif can confer ABA responsiveness to a minimal promoter (Shen et al. 1996). Thus in the case of *OsAKR1*- experimentally determined as the most ABA-responsive- ABRE elements are abundant in the promoter, we could identify a CE3 element as well. The promoter of the other two genes lacks the CE3 motif. This could explain the high ABA inducibility of the *OsAKR1* transcript.

The promoter analysis of the *OsAKR1* gene revealed the presence of three TGACG motifs, which are associated with a variety of transcription factors linked to oxidative stress, auxin, salicylic acid and methyl jasmonate response (Xiang et al. 1996). In addition, *OsAKR1* showed the most prominent response to oxidative stress induced by H_2O_2 treatment. The results of Mylona et al. (2007) give a good supportive evidence for the occurrence of these motifs and ROS response. They demonstrated that the antioxidant genes (coding for CAT, SOD) were upregulated and the antioxidant enzyme activities increased following ROS-inducing xenobiotic treatment in maize. Moreover the TGACG motif was present in all the proximal promoters of these genes induced by ROS. The presence of a given promoter motif might not always be sufficient to induce a stress response. More like the number, order and

type of protein-binding sequences present in promoters are major determinants of the differences in expression patterns of genes (Mahalingam et al. 2003). These support a possible correlation between the high number of TGACG motifs in the *OsAKR1* promoter and the inducibility by oxidative stress.

A new enumeration based motif prediction algorithm (Cserháti et al. 2011) was also applied to compare the number of putative regulatory element dyads found in the promoters of *OsAKR1*, *OsAKR2* and *OsAKR3*. The reported dyad motifs described in the article scored high values in the promoters of the three AKR genes, but the highest value was obtained for the OsAKR1. This again hints to the fact, that the experimentally determined different induction profiles for the 3 AKR genes can be related to the differences in the regulatory elements present in their promoters.

The genes designated *OsAKR1*, *OsAKR2* and *OsAKR3* (NCBI gene annotations: Os01g0847600: *OsAKR1*, Os01g0847700: *OsAKR2* and Os01g0847800: *OsAKR3*), are located in an approx. 39 kbp chromosomal region, together with the *OsP5CS2* gene encoding delta-1-pyrroline-5-carboxylate synthetase, the key enzyme of proline biosynthesis. In order to illustrate their chromosomal position, and the existing homology between the candidate rice AKR genes, we determined the exon-intron boundaries and made a schematic representation of the corresponding genomic region with the neighbouring *OsP5CS2* gene (**Fig. 5b**).

Our choice for this AKR rich chromosomal region was mainly supported by two factors. First is the presented high homology among these three *OsAKR* genes and the previously characterized stress responsive *MsALR* gene from the dicot plant, alfalfa (**Fig. 5a**) The homology tree clearly shows the high similarity between *OsAKR1*, *OsAKR2*, *OsAKR3* and the alfalfa *MsALR*, as well as with the heat inducible *DpARs* isolated from *Digitalis purpurea* (Gavidia et al. 2002). Second, this chromosomal region might be important in the abiotic stress responses (mainly drought and osmotic stress) due to the mapping of different abiotic stress-related QTLs for this chromosomal segment. The identification of drought related QTLs in this region can probably be linked to the presence of the *OsP5CS2* gene (genomic identifier Os01g0848200). The encoded protein is known to be a key regulator of proline biosynthesis, and previous studies on rice have shown that the *OsP5CS2* gene was induced by salt stress, cold and drought stress and by exogenous ABA (Igarashi et al. 1997; Hur et al. 2004).

ABA mediates a variety of physiological processes, including the response to drought and salt stress. Most of the drought-inducible genes studied to date are also induced by ABA and it appears that drought stress triggers the production of ABA directly, which, in turn, induces various genes (Bray 1997; Busk and Pages 1998; Shinozaki and Yamaguchi Shinozaki 2000). Considering that most of those genes responding to drought are also induced by the exogenous ABA (Shinozaki and Yamaguchi Shinozaki 1997), we hypothesize that the induced transcription of *OsAKR1* may also be a part of the abiotic stress adaptation strategy of rice.

5.3. Increased AKR protein synthesis in rice cell suspensions following different stress treatments

In accordance with the stress induced transcriptional responses (**Fig. 6a-c**) a variety of stress agents triggered AKR protein accumulation in rice cell suspensions as well. We could detect significant increase in protein level through Western blotting following H_2O_2 and ABA treatments, but also heat and benzylalcohol (BA) were effective inducers (**Fig. 8a-d**). Several data exists about other plant AKRs also showing stress inducibility in response to different abiotic stress factors; alfalfa AKR protein induced by ABA and hydrogen peroxide (Oberschall et. al 2000), *DpAR* transcript increase following heat shock and drought (Gavidia et. al 2002).

Similarly we could also observe AKR induction following high temperature and BA as well. BA is a high temperature mimicking agent, causing membrane fluidization (Konigshofer et al. 2008). The reason why we used BA as a stress agent was because it imitates the effects of heat stress. There are relatively few plant riports about AKR induction following heat treatment (Gavidia et al. 2002), and no data about that fact, that overexpression of AKR proteins may lead to heat stress tolerance in plants. This is the reason why we chose to proceed in this direction and we were able to demonstrate that transgenic plants overexpressing the OsAKR1 had better photosynthetic parameters; they accumulate less lipid peroxidation derived aldehydes and have higher total AKR activity following heat damage then the wild type plants. In general we can conclude, that the stimulation of AKR synthesis under stress condition points to a physiological role of these enzymes in plants exposed to environmental stresses.

5.4. Enzyme kinetic parameters of OsAKR1

Biochemical characterization revealed that the OsAKR1 enzyme reacts with a variety of toxic aldehydes including MG and MDA. MDA -a major lipid peroxidation marker- is generated in substantial amounts following oxidative stress. The recombinant OsAKR1 accepts MDA as substrate (**Table. 1**), having similar kinetic values as the bovine aldose reductase (Halder et al. 1984). The *in vitro* properties of OsAKR1 against MDA suggest the possibility of a similar *in vivo* action directed towards oxidative stress-linked aldehydes, such as MDA in plants. Constitutively expressed, higher levels of OsAKR1 in the transgenic plants could effectively detoxify MDA beyond a certain level, thus preventing its accumulation to toxic grade. Reduced MDA levels in the transgenic tobaccos (especially B1/1 line) compared to SR1 plants following MV treatment are strenghtening this hypothesis. Similarities could be observed in the kinetic constants of OsAKR1, alfalfa AKR and human aldose-aldehyde reductase as well for a number of substrates (Bohren et al. 1991; Oberschall et al. 2000). The high kcat/Km value of the OsAKR1 enzyme to MG makes the efficient functioning of this enzyme feasible under *in vivo* conditions as well.

We proposed the enzymatic characterization of the other two AKR proteins as well (OsAKR2 and OsAKR3), but despite numerous attempts it proved to be unsuccesfull. Our initial trial was the most commonly used and easiest method, the prokaryotic expression system (*E. coli*), where we could get considerably high yield of soluble, yet inactive recombinant proteins. Several optimization steps have been tried in order to get active proteins, (lowering the temperature, low IPTG concentration, using different *E.coli* strains and epitope tags) yet all ended in failure. Our further attempt was the use of a cell-free wheat germ extract based *in vitro* translation system; this too yielding negative result. Thus both the prokaryotic and eukaryotic expression system did not result in the enzymatically active protein. There could be a few reasons behind this fact; one of the plausible explanations would be, that the obtained proteins were actually active, but have such a narrow substrate specificity that we were unable to identify. However, a more straightforward explanation can be provided, by examining their predicted secondary structure (**Fig. 5d**). We can see differences in the position of the alpha helices in the three OsAKRs especially in the regions involved in the cofactor binding.

It has been documented, that all of the AKRs are dependent on a nicotineamide adenine dinucleotide cofactor for redox chemistry and kinetic analysis have revealed, that AKRs follow an ordered reaction mechanism, where the NAD(P)H cofactor binds first and leaves last (Kubiseski et al. 1992). Crystallographic studies revealed that a conformational change appears to be necessary for the NAD(P)H cofactor to gain entrance into and to exit out of the active site (Borhani et al. 1992). Moreover, the NADPH cofactor is a key structural component in organizing both the active site residues and the substrate binding pockets to produce a catalytically competent enzyme (Sanli and Blaber 2001). Taking these into account we can hypothesize, that the conformational change allowing the cofactor to access the active site might be more complicated if the alpha helices are situated in the region where the cofactor is supposed to bind. By looking at the secondary structure we realize, that OsAKR2 and OsAKR3 are more similar to each other then to OsAKR1. These differences in the position of alpha helices suggest that the difficulty of proper folding was higher in the case of the OsAKR2 and OsAKR3 recombinant proteins, thus increasing the chance for the production of catalytically inactive enzymes. Summarizing, we can propose, that either differences in the enzyme structures or the small possibility of finding the proper substrates could be the reasons behind the unsuccessful results in the enzymatic characterization of OsAKR2 and OsAKR3.

5. 5. OsAKR1 plays important role in reactive carbonyl detoxification *in vivo*

High concentrations of MG are toxic for the cells as it inhibits cell proliferation (Ray et al. 1994) and results in a number of side-effects, such as increasing the degradation of proteins by modifying Arg, Lys, and Cys residues (Martins et al. 2001). Therefore, it is quite important to effectively disarm or prevent the damaging effects of MG accumulation. Our enzyme activity measurements showed, that the OsAKR1 enzyme could accept MG as a substrate (**Table 1**), as well as its ability to detoxify MG even in bacterial cell culture (**Fig. 12**). Similar data was reported for a bacterial AKR (*E. coli YgHZ*). Overexpression of this enzyme resulted in tolerance of *Escherichia coli* cells against increasing concentrations (1-5 mM) of MG (Grant et al. 2003). Additionally the transgenic tobacco lines overproducing OsAKR1 showed higher resistance to 10 mM MG than the wild type plants. The ability of the transgenic plants

to maintain their photosynthetic performance for longer time despite the damage caused by this cytotoxic agent was a good indicator in the overall determination of the plant condition. MG has been suggested as moderately toxic in the inactivation of photosynthetic CO_2-photoreduction, used in 2 mM concentration, as compared to 2-alkenals (Mano et al. 2009), nevertheless in higher concentrations the effect might be more toxic. As reflected by the photochemical yield, the B1/1, D3/3 and D3/2 lines performed better after 18 hr exposure to a considerably high, 10 mM MG concentration (**Fig. 14**). Probably the protective effect of OsAKR1 overproduction in photosynthesis manifests on the level of reactive carbonyl detoxification, generated as a consequence of exaggerating MG amounts. However the ability of OsAKR1 to detoxify MG both in bacterial and plant system is important in demonstrating the highly conserved function of AKR proteins: the produced rice protein can perform its reactive aldehyde scavenging duty *in vivo* within *E. coli*, and tobacco as well by complementing the action of the endogenous AKR proteins.

5. 6. *In vitro* modification and inactivation of PRK by MDA and MG

PRK is a key enzyme of the Calvin cycle, catalyzing the ATP-dependent phosphorylation of ribulose-5-phosphate to ribulose-1,5-phosphate. Being an enzyme regulated by the reversible oxidation/reduction of sulfhydryl and disulphide groups it can be a plausible target for reactive aldehyde modification in the chloroplasts, due to the presence of cysteins in the active domain.

In the case of prolonged stress conditions, the potential of antioxidant defense system of an organism (including plants) can be depleted, which results in a series of metabolic outcomes; for example the accumulation of hydroxyl-radical induced lipid hydroperoxidation products and reactive aldehydes that are subject to Michael addition reactions with the side chains of lysine, histidine and cystein residues, referred to as protein carbonylation (Grimsrud et al. 2008). Peroxides of polyunsaturated fatty acids generate malondialdehyde (MDA) on decomposition, and in many cases MDA is the most abundant individual aldehydic lipid breakdown product (Esterbauer and Cheeseman 1990). It has been estimated that more than 75 % of the measured MDA is derived from triunsaturated fatty acids such as linoleic acid (18:3) (Weber et al. 2004). *In vivo*, MDA and other aldehydes can alter proteins, DNA, RNA, and

other biomolecules through addition reactions with Schiff's bases (Esterbauer and Cheeseman 1990) but they also function as powerful secondary messenger's upregulating many genes implicated in the plant (a)biotic stress response (Esterbauer et al. 1991, Vollenweider et al. 2000, Farmer et al. 2003, Weber et al. 2004).

Contrary to MDA which is lipid peroxidation-derived, MG is mainly a by-product of glycolysis and it is formed spontaneously from the triose-phosphates. As a highly reactive physiological metabolite it forms adducts with basic amino acid side chains, referred to as AGE products. MG reacts predominantly with arginine to form – among other derivatives – hydroimidazolones and arg-pyrimidine (Ahmed and Thornalley 2003). Reactivity with cysteine and lysine residues to form derivatives such as N-(carboxyethyl) lysine is less pronounced (Grillo and Colombatto 2008). Moreover, there is increasing evidence that MG modifications can alter physiological properties of proteins (Morgan et al. 2002).

In our case the activity of PRK was severely inhibited by exposure to both 1 mM MDA and 5 mM MG after 1 hrs treatment time (**Fig. 11d, e**). Immunodetection revealed that the formation of MDA adducts is in direct correlation with the incubation time (**Fig 11a**). Burcham and Kuhan (1996) showed, that bovine serum albumin (BSA) incubated with MDA resulted in a time- and concentration-dependent increase in the carbonyl content detectable by spectrophotometric or immunochemical assays. They also found, that the highest level of carbonylation was observed at pH 4, but the reactivity of MDA at physiological pH was also enough to confer toxicological significance. MDA primarily reacts with the amino groups of lysine residues, resulting in the chemical denaturation of the proteins by eliminating the positive charge of lysine (Uchida et al. 2000, Ishii et al. 2008). Yamauchi et al. (2010) showed that PSII proteins including OEC33, CP47 and CP43 are the dominant MDA-modified proteins in spinach leaves. Their MDA modifications cause release of OEC33 from PSII, leading to a partial loss of the oxygen-evolving activity. As such, modification of proteins by MDA can impair their normal function thus making its effective detoxification a very important process. The *in vivo* MDA concentrations reported by the literature are 3 nmol (g fresh weight)$^{-1}$ in Arabidopsis (Weber et al. 2004), between 10-16 nmol (g fresh weight) $^{-1}$ in cucumber (Zhang et al. 2008) and 40-250 nmol (g fresh weight) $^{-1}$ for olive leaves and pea leaves (Iturbe-Ormaetxe et al. 1998, Sofo et al. 2004). In the case of cucumber the range is dependent whether it is non-stressed condition (10 nmol) or pathogen infestation (up to 16

nmol). These values can correspond to μM levels in cells when homogenous distribution of aldehydes in the tissue is assumed and can be sub-mM level, when its compartmentation in the cell is considered. Moreover, in the case of stress conditions these concentrations can be even higher. Considering this, the 1 mM MDA used by us for protein modification corresponds to plausible *in vivo* concentration as a result of stress.

MG modification of PRK also resulted in activity decay. In 5 mM concentration, the enzyme activity was almost completely inhibited after 1 hour. Derivatization with DNPH did not show extra protein bands on the membrane (in contrast to MDA) hybridized with the á-DNP antibody (**Fig. 11b**). The results suggest that the modification and inactivation process between the two aldehydes it is different. By using increasing concentrations of MG we could show, that MG adducts can be visible even on the Coomassie gel being in direct correlation with the concentration increase (**Fig. 11c**). MG mediated modification and aggregation of human ceruloplasmin led to enzyme activity loss (Kang et al. 2006). It was also reported, that modification by 10 mM MG changes the physicochemical properties of human serum albumin (HSA) by increasing the molecular weight and decreasing its drug binding properties. In this case the extent of arginine modification was the highest (Mera et al. 2010).

The *in vivo* concentration of MG in several plant species was estimated in the range of 40-75 ìM under non-stressed conditions and up to 200 ìM under salinity stress (Yadav et al. 2005). By using the same method we could also detect concentrations between 200-300 ìM in tobacco under non-stressed conditions (**Fig. 15b**). The real concentrations however could be higher (in the mM range) since it is only possible to measure the concentration of free MG and not the MG which has already reacted with biomolecules. On the other hand the increase in the glycolytic rate under stress conditions could lead to higher MG level as it is mainly produced from triose phosphates (Sommer et al. 2001). Therefore the concentration used by us in this study for protein modification (5 mM) could be considered in the *in vivo* range.

Two enzyme pathways are known to participate in the catabolism of MG. The glyoxalase I-II system, which converts MG to D-lactate via the intermediate *S*-D-lactoylglutathione using reduced glutathione as a cofactor (Thornalley 1998). The other pathway is mediated by AKR's which converts MG to acetol using NADPH as cofactor (Van der Jagt et al. 1992). Under severe oxidative stress conditions the reduced-gluthatione pool of

cells can be exhausted by other antioxidant systems, therefore a substantial role can be granted for the AKRs to detoxify MG.

These experiments were performed in order to illustrate the possible effects of excess aldehyde accumulation on the activity of enzymes (in this case a key enzyme of the Calvin cycle) and to emphasize the role of effective detoxification (e.g. glyoxalase pathway or other alternative routes like overproduction of AKRs). Since both MDA and MG are substrates for OsAKR1, we can assume that its constitutive overexpression helps to detoxify more effectively MG and MDA and therefore preventing their accumulation to toxic rates.

5.7. OsAKR1 improves tolerance to high temperature and oxidative stress in transgenic tobacco

ROS formation as a consequence of abiotic stress is unavoidable. In the vicinity of biomembranes containing polyunsaturated fatty acids this may lead to the formation of lipid hydroperoxides and their toxic aldehyde degradation products, belonging to a group of compounds termed as RCOs. Using MV as a strong oxidative stress-inducing herbicide, we verified the protective role of the OsAKR1 protein in transgenic tobacco lines and provide evidence that OsAKR1 may function as an aldehyde detoxificating enzyme *in vivo*.

Overexpression of OsAKR1 resulted in decreased reactive aldehyde content, as reflected by the reduced accumulation of MDA in the transgenic lines in response to MV (**Fig 16, 17b**). Since MDA proved to be a substrate for OsAKR1 (**Table. 1**) it's highly plausible that this lipid peroxidation-derived toxic aldehyde compound generated indirectly by MV is scavenged by OsAKR1 enzyme *in planta* as well. MV tolerance in transgenic plants has also been reported to increase as the consequence of plants overproducing other reactive carbonyl scavenging enzymes (Mano et al. 2005; Rodrigues et al. 2006). The difference among these enzymes and the AKRs are their mode of action in the common process they catalyze (carbonyl detoxification). Increased stress tolerance has been reported for the overexpression of aldehyde dehydrogenase (ALDHs), enzymes converting the reactive aldehydes to carboxylic acids (Sunkar et al. 2003; Rodrigues et al. 2006), or for the 2-alkenal reductase (AER), reducing their toxicity by double bond saturation (Mano et al. 2005)

The effects of heat stress on photosynthesis are widely documented, but the connection between AKRs and protection against heat induced damage in photosythesis is not yet reported. Photosystem II is heat sensitive (Bukhov et al. 1999), mainly due to the high temperature-induced dissociation of the oxygen evolving complex (OEC), which results in an imbalance between the electron flow from water towards the acceptor side of PSII in the direction of PSI reaction center (DeRonde et al. 2004). Also, heat injury is known to affect the photosynthetic apparatus by deactivating photosystem II, denaturing functional proteins (Thompson et al. 1989) and dissociating the peripherial light-harvesting complexes (Gounaris et al. 1984). In potato leaves kept at 40 °C, the maximum quantum yield gradually decreased, and eventually reached zero after 40 minutes, indicating a complete destruction of PS II (Havaux et al. 1993). Instead of the maximum (potential) PS II quantum yield studied in the above experiment we choose to measure the actual photochemical yield, characteristic of photosynthetic electron transport under the experimental light regime. Following this parameter, we found that the SR1 plants lost approximately half of their photochemical yield as a consequence of the heat treatment. On the other hand, the OsAKR1-overexpressing lines (especially B1/1) were affected to a smaller extent. In fact the B1/1 line exhibited almost 50 % better photosynthetic yield after the heat treatment then the SR1 plants (**Fig. 18**). Here we must emphasize the difference in the accumulated OsAKR1 protein amounts in the transgenic lines, with B1/1 synthesising the most.

In order to pursue further how the mechanism of tolerance relates to the higher protein accumulation in the transgenic lines, we aimed to look for a possible overlap between the photosynthetic performance and the putative role played by the OsAKR1 in this phenomenon. We compared the AKR activity of leaf extracts before and after the heat treatment. These results showed that the overexpressing lines present a significantly higher AKR activity already in the control stage, prior to heat stress then the SR1 (**Fig. 19**). Thus this elevated activity cannot be attributed to heat exposure, but presumably to an already existing higher AKR background, resulting from the overexpression of the transgene. Obviously, the omnipresence of a higher supply of scavenging enzyme in the transgenic lines makes the cells better-armed for the toxic effects of heat stress, as compared to the lines where there is no overexpression. Furthermore, the observed AKR activity of the leaf extracts is also correlating with the OsAKR1 protein amounts detected in these transgenic lines (**Fig. 13**), thus supporting

the previous statement. These results originating from leaf disc assays suggest that the actual amounts of OsAKR1 protein in the transgenic plants could be associated with their better photosynthetic performance following heat treatment, suggesting a protective function of OsAKR1. This can be attributed again to the ability of certain AKRs to act against lipid peroxidation products.

In wheat thylakoids an increased rate of lipid peroxidation was reported following heat stress, making the components of the electron transport chain more susceptible to photoinhibition. This insinuates that lipids are required to sustain normal photosynthetic activity under environmental stress. Beyond this, the formation of excessive amounts of lipid peroxidation products (such as MDA), can lead to the complete loss of the photosynthetic activity (Mishra and Singhal 1992). The results of Yamauchi et al. (2010) on heat stressed plants demonstrated that MDA content in leaves increased by more then 3 fold after only 2 hrs on 40 ºC. Moreover, a loss of oxygen evolving ability was observed in the thylakoid membranes treated with MDA at 40 ºC, along with a decrease in the maximal efficiency of PS II. Among chloroplast proteins, the OEC33 and LHCII were modified by MDA (Yamauchi et al. 2008). Thus it seems that MDA accumulation coupled with high temperature stress can have a negative impact on the photosynthetic functions. It is worth mentioning, that on 25 ºC, the oxygen evolution of the thylakoid membrane was not inhibited with 1 mM MDA (Yamauchi et al. 2010) and neither were the stromal photosynthetic reactions by 2 mM MDA treatment at the same temperature (Mano et al. 2009). It seems that MDA is toxic at high temperature, which was shown by the release of OEC33 from the core of PSII at 40 ºC, coupled with MDA treatment (Yamauchi et al. 2010). Based on these data the decreased photochemical yield following heat stress in the wild type plants may be the consequence of excess MDA accumulation and the ability of MDA to impair the photosynthetic function at high temperature.

The results of Yamauchi et al. (2008) on heat stressed plants clearly demonstrated, that the production of reactive compounds from peroxidized linolenic acid play a major role in the damage of the proteins of the photosynthetic machinery. Based on these findings the decreased photochemical yield following heat stress may be the consequence of excess reactive aldehyde accumulation. Indeed the *OsAKR1* overexpressing lines that produce the reactive aldehyde detoxifying enzyme could reserve their photosynthetic functions more efficiently after heat

stress compared to the control plants, which hints to the protective function of this enzyme against high temperature induced lipid peroxidation products.

5. 8. OsAKR1 overexpression leads to reduced MDA levels following heat stress and lower MG content in the control conditions

Whether or not high temperature increases the level of certain aldehydes (particularly MDA and MG) was the question needed to be answered in order to make a conjunction between OsAKR1 overproduction and reactive carbonyl mediated heat stress damage. We compared the levels of MDA and MG before and after heat treatment between the transgenic lines and the SR1. As represented by **Fig. 15a** there was a slight increase in the amount of MDA following high temperature stress in the wild type plants, on the other hand the transgenic lines showed significantly lower levels of MDA following heat damage then the control plants. This supports the earlier hypothesis; that the lower levels of MDA detected in the transgenic lines following high temperature and the better photosynthetic performance of the same lines after heat stress might be due to the prevention of MDA-mediated photosynthetic damage through a more efficient detoxification by the constitutive overexpression of the *OsAKR1* transgene.

ROS scavenging mechanisms have a well-defined role in protecting plants against high temperature and/or high light induced damages (Larkindale and Knight 2002, Suzuki and Mittler 2006). The importance of effective ROS detoxification implies that getting rid of ROS generated peroxidation products is equally important in the maintenance of cellular integrity against cytotoxic agents. MDA increase following heat treatment was well documented in *Arabidopsis thaliana* (Larkindale and Knight 2002). The authors concluded that MDA levels were highest after 3 days recovery following heat stress, under illuminated conditions. Accordingly, the generation of lipid-peroxidation mediated TBARS is not an early, immediate effect of heat exposure. This coincides with our observations. The reason behind it could be the gradual decrease in the antioxidant enzyme activity as demonstrated by Gong et al. (1998). Constitutive overexpression of OsAKR1 caused reduced levels of MDA in the transgenic tobacco plants, whereas in the wild type a slight increase was observed compared to control. This proves the possible benefits of a single gene approach, where the overexpression of a

detoxifying enzyme might help in acquiring higher resistance for transgenic plants to a broad spectrum of stresses (heat, MV), that are linked to the formation of ROS and ROS-mediated peroxidation products.

Contrary to MDA, MG levels did not change before or after heat stress. It looks as if the level of this glycolytic-originated dicarbonyl compound is not influenced by high temperature, at least in this experimental system. We have measured the levels of MG, before and after heat damage, however in this case heat stress did not cause an increase in free MG level. However, there is an interesting point to be mentioned here; in the SR1 plants we could measure almost three times more MG already in control stage that in the B1/1 line (**Fig. 15b**). The other two lines (D3/2 and D3/3) had also significantly lower MG levels compared to SR1. Following heat damage, this profile remained the same, suggesting that overexpression of OsAKR1 might help in regulating the level of this cytotoxic product, already under non-stressed conditions, probably by completing the effect of the main MG detoxification route, the glyoxalase pathway. The constitutive overproduction of a detoxifying enzyme has the advantage to represent a safety stock perpetually present in the cells, ready to be used for different processes. In this case of a metabolite such as the MG, being an attendant of the glycolysis and generated in substantial amounts even under normal conditions, auxiliary pathways involving the AKRs are important to quickly and more effectively metabolize this harmful product and to support other detoxification pathways.

It is not surprising to see, that the patterns for MDA- and MG-levels in the wild type and transgenic plants are different in this experimental setup. This can be linked to the lipid peroxidation mediated origin of MDA, in contrast to MG, which is mainly of glycolytic provenance (Richard 1991). The AKR activity of total extracts correlates with the OsAKR1 protein level detected by Western blot in the transgenic lines (**Fig. 13**) and with the results obtained for MG levels (**Fig. 15b**). B1/1 line showed the highest AKR activity, and lowest MG level. Also the B1/1 line performed better following MV stress, heat damage and MG treatment.

It is difficult to see through all the metabolic changes that occur, or entire processes surrounding a single gene manipulation. It is equally challenging to predict exactly at which level the overproduction of a protein will exercise its effects. There are a number of approaches to increase stress tolerance by manipulating the expression of endogenous stress-

related genes. Strategies targeting transcription factor expression have been shown to be effective due to the consequent up-regulation of many downstream genes (Kasuga et al. 1999; Hsieh et al. 2002). Stress tolerance can also be achieved by changing the expression of a single gene (Zhu 2001; Kumar et al. 2004) by either preventing the intracellular stress build-up, or accelerating the detoxifying processes and cell repair through the clearance of harmful products (Rodrigues et al. 2006). **Fig. 20** summarizes the connections between AKRs and the main metabolic pathways that are related to the generation of ROS and ROS-mediated toxic products in the plant cell.

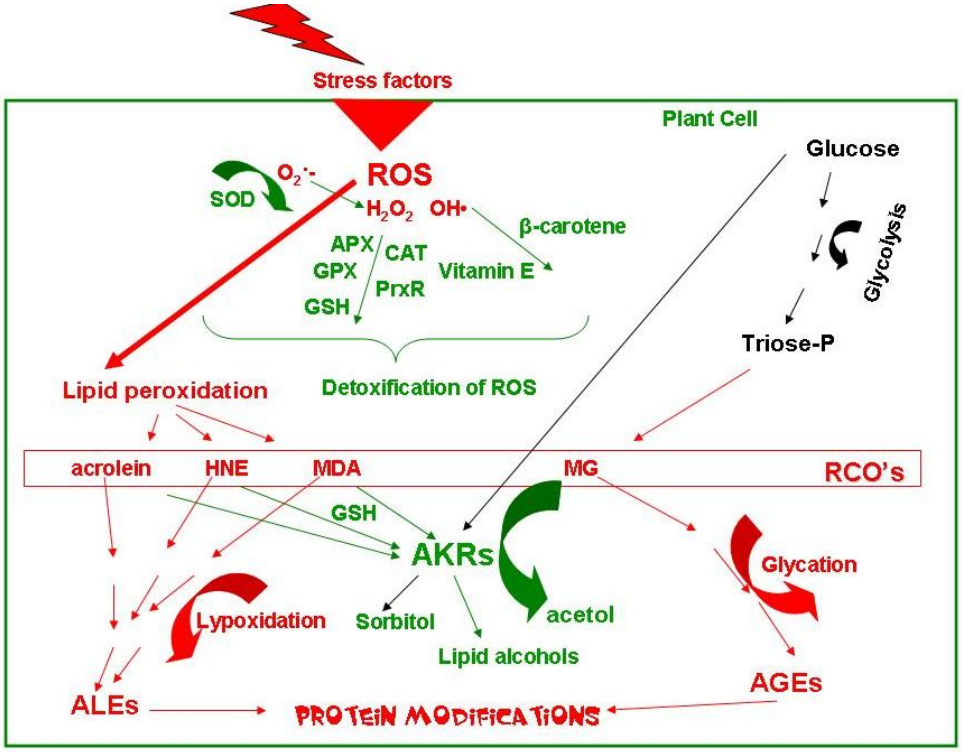


Figure 20. Schematic overall representation of the stress induced ROS-production and different detoxification mechanisms, complemented by the AKRs in a plant cell. Red arrows indicate metabolic steps that lead to the formation of toxic compunds and red names mark the toxic compounds. Green arrows and names indicate scavenging pathways and detoxifying molecules. The number of arrows do not exactly represent the number of metabolic steps and serve an illustrative purpose only. (Triose-P (triose-phosphate, GSH-reduced gluthatione)

The AKRs, by complementing the antioxidant enzyme supply, have a distinctive role in the clearance of either lipid-peroxidation- or glycolysis-derived RCOs. In the absence of proper detoxification strategies, the accumulation of these compounds eventually lead to the

formation of AGEs and ALEs and can result in irreversible protein modifications, presenting a serious threat to the cellular homeostasis. According to our hypothesis constitutive overproduction of OsAKR1 is mainly beneficial in the detoxification processes against the reactive aldehydes generated at increased levels under stress. This statement is backed up by a series of indirect evidence involving processes that are linked to these enzymes (enzyme kinetic features, reactive carbonyl detoxification, stress-inducibility etc.). In summary, our results support, that OsAKR1 may be an effective engineering target to improve abiotic stress resistance of agriculturally important crop plants through the increase of the activity of reactive carbonyl detoxification pathways.

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8. SUMMARY

Introduction

The bioproductivity of plants largely depends on their equilibrium with the environment and the developement of large scale adaptation processes to different stress factors. Plants exposed to biotic or abiotic stress are subjected to oxidative 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 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 (Oberschall et. al 2000).

Plant AKRs, among other enzymes, have been shown to be effective in the detoxification of lipid peroxidation-derived reactive aldehydes (Hideg et al. 2003; Mundree et al. 2000; Oberschall et al. 2000). The first isolated AKR homologue protein from barley showed osmoprotective function during embryo development and was linked to the acquisition of desiccation tolerance (Bartels et al. 1991; Roncarati et al. 1995). Moreover, a protein designated as ALDRXV4, member of the AKR superfamily, was found to be present in high amounts in the dehydrated leaves of the ressurrection plant *Xerophyta viscosa* along with elevated levels of sorbitol as osmoprotectant (Mundree et al. 2000). The recently characterized AKR4C9 (*At2g37770*) from *Arabidopsis thaliana* (Simpson et al. 2009) supports the importance of the enzymatic action directed towards oxidative stress-linked aldehydes, such as malondialdehyde (MDA). A set of experimental data indicated that transgenic tobacco plants overexpressing an alfalfa AKR protein (MsALR), showed 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 (Hegedűs et al. 2004; Hideg et al. 2003, Oberschall et al. 2000).

Aims of study

Initially we were looking for *AKR* genes involved in abiotic stress responses by analyzing the fully sequenced genome of the monocot crop plant, rice. After the identification of the candidate stress responsive, AKRs by the abundance of stress responsive elements in their promoter and by their transcriptional response to a series of stress treatments, we proposed to analyze both *in vitro* and *in vivo* the reactive carbonyl scavenging capacity of the encoded proteins. Finally, our aim was to overproduce these proteins in transgenic plants to create and analyze genotypes, which will have a broad spectra of reactive carbonyl-mediated stress-resistance.

We have selected a short chromosomal region containing three *AKR* genes. This part of chromosome 1 also contains the P5CS2 gene, encoding the key enzyme of proline biosynthesis; suggesting the importance of this chromosomal region in abiotic stress response. The *AKR* genes showed high homology with the previously characterized stress responsive dicot *MsALR* gene. Among the three rice AKRs tested, the expression of *OsAKR1 (Os01g0847600)* gene was greatly induced by abscisic acid (ABA) and H_2O_2. The *OsAKR2 (Os01g0847700)* and *OsAKR3 (Os01g0847800)* genes responded with modest transcriptional changes to these treatments. To complement the transcript analysis we proposed to monitor the changes in the endogenous rice AKR protein levels as a response to different abiotic stress treatments (BA, heat stress, ABA, H_2O_2) Further studies on the purified OsAKR1 recombinant protein showed strong enzymatic affinity toward several harmful, lipid degradation or glycolysis-derived aldehydes (e.g, MG, MDA) and to sugar substrates also. The overproduction of this enzyme in *Esherichia coli* increased the *in vivo* tolerance of bacterial cells against MG and the overexpression *in planta* led to decreased reactive aldehyde levels (MDA and MG). Stress-induced, excessive accumulation of MDA and MG is toxic due to their ability to oxidatively modify proteins. We aimed to demonstrate this *in vitro*, with recombinant protein. We chose a key enzyme of the Calvin cycle (PRK) which is prone to protein carbonylation due to the presence of cysteins in the active domain. Incubation of this recombinant enzyme with 1 mM MDA and 5 mM MG resulted in rapid decrease in the enzymatic activity along with massive protein carbonylation detected by immunochemical methods. This example shows that it is important to effectively detoxify and prevent the

accumulation of these toxic compounds. In the frame of this concept we aimed to create stress resistant transgenic tobaccos through the heterologous overproduction of OsAKR1. The transgenic plants showed improved tolerance to MV and heat treatment in leaf disc assays. Following heat stress the assayed overexpressing plants contained less MDA and MG then the wild type. Ultimately the final purpose of this research is to implement this method to obtain stress resistant crop genotypes with agricultural importance.

Results and short discussion

Our work started with the search for highly homologous rice AKR genes with the previously characterized stress inducible alfalfa *MsALR* (Oberschall et al. 2000) and the recently characterized At2g37770 from Arabidospis (Simpson el al. 2009). As a result we identified a chromosomal region rich in AKRs in the close proximity of the abiotic stress-inducible *P5CS2* gene, suggesting that this particular locus might have role in the stress adaptation. The three AKR genes located in this region of the *Chr1* presented high homology with several well-characterized plant AKRs. 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. 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 ABREs (ABA-responsive element), CE3 (coupling element associated with ABA response) and motif IIb (abscisic acid responsive element) among the three rice AKRs, which is positively correlating with the transcriptional response given for ABA treatment.

AKR protein induction following stress was also demonstrated in rice cell suspensions. Similarly to the transcriptomic changes, stress treatments like heat, ABA, H_2O_2 and BA

induced AKR protein accumulation. Based on the transcript profiling and the abundance of stress responsive promoter elements we selected OsAKR1 for further analysis. The enzymatic characterization revealed that OsAKR1 accepts a variety of lipid peroxidation and/or glycolysis derived aldehydes as substrates, along with some sugars also. For the other two AKR proteins we could not show any detectable activity on any of the substrates we tested, probably because of the differences in the secondary structure or a very narrow substrate preference. 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 affinity for OsAKR1 towards MDA is represented with a Km value that is much closer to its predicted *in vivo* concentration than it was reported for an *Arabidopsis thaliana* aldo-keto reductase (Simpson et al. 2009).

Both MDA and MG are protein modifying agents, which exert their action (among other ways) through a mechanism called protein carbonylation. The attachment of carbonyl residues on amino acid side chains usually results in the loss of function for the protein. We have shown the negative effects of protein carbonylation by these aldehydes on a Calvin cycle enzyme (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. This shows just how important is the detoxification of these aldehydes before their concentration reaches excessive levels.

Heterologous overexpression of OsAKR1 in tobacco resulted in better photosynthetic performance after heat stress and reduced MDA content compared to the wild type plants. The transgenic plants also showed less MDA levels after the oxidative stress imposed through MV treatment. In general 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. MG content did not increase after heat stress contrary to MDA. This observation can be attributed to the different pathways playing role in the generation of MDA and MG. MDA is mainly lipid-peroxidation derived and formed from linolenic acid while MG formation is linked to the glycolysis and triose phosphates. It seems that MDA is toxic at high temperature, which was shown by the release of OEC33 from the

core of PSII at 40 ºC, coupled with MDA treatment (Yamauchi et al. 2010). On 25 ºC, the oxygen evolution of the thylakoid membrane was not inhibited with 1 mM MDA (Yamauchi et al. 2010) and neither were the stromal photosynthetic reactions by 2 mM MDA treatment at the same temperature (Mano et al. 2009).

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 of the most important cereal crop plant can help to establish *cis*-genic approach to create abiotic stress tolerant rice.

9. ÖSSZEFOGLALÁS

Bevezetés

A környezeti stresszhatások nagymértékben megzavarhatják az asszimilációs és az oxidatív folyamatok közti egyensúlyt, ami gyakran a fotoszintézis mértékenek a csökkenésében és reaktív oxigénformák felhalmozódásában nyilvánul meg. Ez utóbbinak egyenes következménye lehet a különböző biomolekulákkal történő interakciójuk (lipidperoxidáció, fehérjék, DNS károsítása) és az így keletkező citotoxikus, öregedést elősegítő anyagok akkumulációja.

Az aldo-keto reduktáz (AKR) enzimcsalád egyik klasszikus tagját, az aldóz reduktázt, a poliol anyagcsere út első enzimeként azonosították, amely a glükóz-szorbitol átalakulást katalizálja. A cukroknak cukor-alkoholokká történő redukálásán kívül egyes AKR-ek hatékonyan redukálnak számos toxikus, lipidperoxidációból és glikolízisből származó aldehideket (pld. malondialdehid (MDA), 4-hidroxinon-enál, metilglioxál (MG)). Ezáltal védik a sejteket az öregedési folyamatoktól, de ugyanakkor fontos ozmolitképzők is. Növényeknél az ozmoreguláció fontos szerepet játszik a szárazságtűrésben (Mundree et al. 2000).

Ugyancsak a növényi szakirodalomból jól ismert az aldóz reduktázoknak a mag kiszáradásakor betöltött ozmoprotektív szerepe (Bartels et al. 1991), valamint a különböző xenobiotikus aldehideket méregtelenítő funkciója (Kolb et al. 1994; Colrat et al. 1999). Nemrég, egy AKR4C9 (*At2g37770*) névvel illetett AKR-t azonosítottak az *Arabidopsis thaliana*-ból (Simpson et al. 2009) és kimutatták, hogy a rekombináns fehérje szubsztátjai között szerepel az egyik fő lipid-peroxidációs termék, a malondialdehid (MDA) is. A lucerna *(Medicago sativa)* AKR-t túltermelő dohánynövényeknél is magasabb fokú rezisztenciát tapasztaltak a metilviologén (MV), nehézfémek, UV-B sugárzás által indukált oxidatív károsodással szemben, mint a vad típusú növényeknél (Oberschall et al. 2000, Hideg et al. 2003, Hegedűs et al. 2004).

Célkitűzések

Kutatásunk átfogó célja rizs AKR-ek azonosítása, izolálása és jellemzése, illetve felhasználásuk a transzgénikus technológia segítségével stressztoleránsabb rizs- és búza genotípusok létrehozására. *In silico* predikciók (cDNS könyvtárakból származó génexpressziós adatok, promóteranalízis, homológia keresések, kromoszómális lokalizáció) alapján kiválasztottunk három rizs *AKR* gént. Ezek a rizs I. kromoszómáján, egymás szomszédságában talalható *AKR* gének a már részletesen jellemzett, abiotikus stresszekre indukálódó, a prolin bioszintézisében kulcsfontosságú szerepet jatszó fehérjét kódoló génhez közel helyezkednek el. A három rizs *AKR* gén kifejeződésének a változását vizsgáltuk különböző stresszkezelések hatására (abszcizinsav (ABA), H_2O_2, NaCl és mannitol kezelt rizs sejtszuszpenzióban) kvantitatív Real Time-PCR segítségével. Hasonlóképp célkitűzésként szerepelt a rizs sejtszuszpenziókban különböző abiotikus stresszorok (hőstressz, benzil-alkohol, ABA, H_2O_2) hatására az AKR fehérjeindukcó megvizsgálása. A stresszhatások következtében kialakult expressziós mintázat alapján az *OsAKR1* gén mutatott a legmagasabb indukciót az előbb említett kezelésekre.

Ebből kiindulva további célkitűzésként szerepelt az OsAKR1 rekombináns fehérjének az enzimkinetikai paramétereinek a vizsgálata, illetve *in vivo* és *in vitro* kísérletekben meghatározni a különböző toxikus aldehidek méregtelenítési hatékonyságát. Végül pedig a célkitűzéseink fontos részét képezte e fehérjének a transzgénikus dohányokban történő overexpresszió es a túltermelő vonalak stressztűrésének a vizsgálata es összehasonlítása a vad típusú növényekével. Ilyen stresszhatások voltak a magas hőmérséklet és az oxidatív stressz.

E kísérletek mintegy előfutárát képezik majd azoknak a cisz- illetve transzgénikus próbálkozásoknak, amikor egy rizsből származó reaktív karbonil detoxifikáló enzimnek a túltermeltetése jobb stressztűrést biztosítana gazdaságilag fontos növényeknek (pld. rizs, árpa, búza). Az erre vonatkozó genetikai transzformációs lépések már megtörtentek, a transzgénikus növények jellemzése hamarosan elkezdődik.

Eredmények és rövid diszkusszió

A génexpressziós mintázatot vizsgálva kiderült, hogy a számos stresszreakció lefolyásában közvetítő szerepet játszó ABA hatására több mint 10-szeres transzkriptum növekedést lehetett tapasztalni a kontrollhoz képest az *OsAKR1*-nél, de ez a gén az ozmotikus illetve sóstresszre is jól indukálódott. A kiemelkedő indukciós profil a gén promóterében található promóterelemekkel lehet összhangban. Azt találtuk, hogy az *OsAKR1* promótere tartalmazta a legtöbb ABRE es TGACG központi motivumot tartalmazó promóterelemet, amelyek szerepet játszanak az ABA és az oxidatív stresszválaszok kialakításában. Ezen kívül egy új enumerációs algoritmus segítségével is analizáltuk a promótereket. Ebből is az derült ki, hogy az előbb említett három gén közül az *OsAKR1* tartalmaz a legtöbb, stresszindukcióban részt vevő transzkripciós faktor kötőhelyet. Ezek alapján az *OsAKR1* gén lett a „kiválasztott", a kódoló szekvenciáját amplifikáltuk és bakteriális expressziós vektorba klónoztuk rekombináns fehérjetermeltetés céljából. Az *Escherichia coli*-ból tisztított rekombináns GST-OsAKR1 fehérjének *in vitro* megmértük az enzimaktivitását számos, toxikus aldehid és néhány cukor szubsztráton. Kiderült, hogy az enzim nagy hatásfokkal metabolizál olyan toxikus- és normális élettani körülmények között is nagy mennyiségben keletkező aldehideket, mint a MG és MDA, valamint néhány cukor szubsztrátot is. A másik két rizs *AKR* gén (*OsAKR2, OsAKR3*) által kódolt fehérjéken nem sikerült aktivitást kimutatni. Ennek legvalószínűbb oka a fehérjék másodlagos szerkezetben észlelt különbségek, de lehet az is, hogy annyira szűk szubsztrátspecificitással rendelkeznek, hogy nem sikerült ezeket azonosítani.

Az MG egy nagyon reaktív oxo-aldehid, ami a glikolízis melléktermékeként nagy mennyiségben képződik (Jan et al. 2005). Az MDA egy lipid-peroxidációs termék és főleg linolénsavból keletkezik. Mindkét aldehid toxikus és jól ismert karbonilációs képességéről, amely módosítás általában a fehérjék funkciójának az elvesztését is jelenti. Kísérletileg igazoltuk, hogy az 5 mM MG és 1 mM MDA képes majdnem teljes mértekben inaktiválni olyan fehérjéket, mint pld. a Calvin-Benson ciklus egyik kulcsenzimét, a foszforibulokinázt (PRK).

Az *Arabidopsis thaliana*-ból klónozott rekombináns PRK az aktív centrumban található ciszteinek jelenléte miatt érzékeny lehet a reaktív aldehidek által okozott fehérjemódosításokra. A kísérlet célja annak az igazolása volt, hogy egy biológiailag fontos szerepet betöltő enzim nagyon könnyen inaktiválodthat, ha a környezetében a reaktív aldehid koncentráció megnő és nem hatékony a detoxifikálás. A fő MG detoxifikáló enzimrendszer a glioxaláz I-II (Singla Pareek et al. 2003), de ehhez redukált glutation szükséges, ezért ha kimerül ez a tartalék, akkor az AKR-ekre is fontos szerep járul a méregtelenítésben. Említésre méltó, hogy mindkét reaktív aldehid szubsztrátja az OsAKR1-nek, ezért egy ilyen méregtelenítő fehérjének a konstitutív túltermeltetése megoldás lehet e toxikus aldehidek által okozott fehérjemódosításoknak a megelőzésében.

In vivo is sikerült kimutatni az OsAKR1 fehérje MG detoxifikáló hatását bakteriális rendszerben illetve OsAKR1-et túltermelő dohánynövényekben is. Azok a baktériumsejtek, amelyek termelték a rekombináns AKR fehérjét jobban tolerálták a magas MG koncentrációt, mint a kontroll sejtek. A transzgénikus dohányok esetében a túltermelő vonalak jobb fotoszintetikus paraméterekkel rendelkeztek, mint a vad típus 10 mM MG kezelés után, ami hatékonyabb reaktív karbonil detoxifikációra utal. Az OsAKR1 fehérjének e hatásának a megnyilvánulása mind bakteriális mind pedig növényi rendszerben egy konzervált funkcióra utal. A teljeskörű jellemzés elengedhetetlen feltétele volt, hogy megvizsgáljuk a gén és a fehérje viselkedését transzgénikus növényekben. Így növényi expressziós vektorba klónoztuk a célgént, ahol kifejeződését a konstitutív 35S CaMV promóter biztosította, majd *Agrobacterium tumefaciens* közvetítésével transzformáltuk dohányba (*Nicotiana tabacum*). A különböző generációs (T2, T3) szegregáló dohányvonalakat a termelt rekombináns fehérjeszint alapján Western hibridizációval azonosítottuk, és eszerint válogattuk ki a magasan expresszáló vonalakat (B1/1, D3/2, D3/3).

Eredményeink alapján az OsAKR1-et túltermelő dohányvonalakban (főleg a magasan expresszáló B1/1 vonal esetében) kisebb MDA koncentrációkat lehetett kimutatni 100 µM MV okozta erős oxidatív károsodást követően mint a vad típusú növényekben, ami hatékonyabb detoxifikációra utal. Hőstressz alkalmazása után a transzgénikus levélkorongok fotoszintetikus hozama jóval meghaladta a vad típusúakét. Pozitív korrelációt találtunk a transzgénikus vonalakban megtalálható rekombináns fehérjeszint és a stressztűrések mértéke között, ami összefüggést jelez a két folyamat között. Talán az OsAKR1 túltermeltetéséből adódóan

hatékonyabb ezekben a növényekben a hőstressz során akkumulálódó reaktív karbonilok detoxifikációja. A túltermelő dohányonalakban az AKR aktivitás magasabb volt, mint a vad típusban, illetve a hőstresszt követően kevesebb lipid peroxidációs terméket (MDA) sikerült kimutatni bennük, mint az SR1-ben. Az endogén MG szint is kisebb volt a transzgénikus dohányokban, ami arra utal, hogy e detoxifikáló enzimnek a túltermeltetése jól kiegészíti a fő MG semlegesítő útvonalat: a glioxaláz rendszert.

Jelenleg folyik az *OsAKR1*-el transzformált búzavonalak vizsgálata, valamint az *OsAKR1* gén promóterének tesztelése riportergénes konstrukcióban. A stressztűrésben szerepet játszó gének feltérképezése a hagyományos nemesítési eljárásokkal karöltve, rezisztensebb genotípusok létrehozása törekszik, főleg a gazdasági haszonnövények körében és ehhez a hasonló kutatások, jó kiindulási alapot szolgáltatnak.