Aldose reductase in rice (*Oryza sativa* L.): stress response and developmental specificity

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Abstract

Aldose reductase (AR) protein and enzyme (alditol: NAD (P)^+ 1-oxidoreductase, EC 1.1.1.21) activity have been identified in mature seeds of *indica* rice cultivars. The protein begins to accumulate 15 days after pollination, reaches a peak at seed maturity and disappears upon imbibition. Furthermore, AR is induced in vegetative tissues in response to exogenous ABA application and other stress conditions, such as PEG mediated water stress and salinity. Increase in AR protein levels upon stress are in close agreement with a similar increase in enzyme activity. Varietal differences in AR levels have been demonstrated. Interestingly, all tested tolerant cultivars (as denoted by breeders) accumulate AR in vegetative tissue in response to ABA application, while the sensitive line, Hamsa, does not do this under similar stress conditions, suggesting that AR may be associated with stress tolerance. Furthermore, AR protein has been identified in mature seeds of some selected cereals indicating the conserved nature of AR across grasses. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Aldose (aldehyde) reductases (AR) belong to the well-conserved aldo–keto reductase super family of enzymes in plants and animals [1–3]. They (alditol: NAD (P)^+ 1-oxidoreductase, EC 1.1.1.21) are monomeric, cytosolic proteins that catalyze the NADPH dependent reduction of a variety of carbonyl metabolites including the aldehyde form of glucose which is subsequently reduced to the corresponding sugar alcohol, sorbitol [4]. They are reported to have broad substrate specificities. For instance, AR from *Euonymus japonica* was reported to reduce a range of substrates including aldos, aliphatic and aromatic aldehydes [5]. AR have been implicated in different cellular processes such as desiccation tolerance of maturing seed and vegetative tissues [2,6], and detoxification of biogenic and xenobiotic aldehydes to their corresponding alcohols in plant and mammalian tissues [3,7–9]. AR in mung bean detoxifies the fungal toxin eutypine [3] and a carbonyl reductase metabolizes HC toxin that affects sensitive maize cultivars [7]. In mammalian cells, AR are reported to be involved in the inactivation of various toxic aldehydes such as chlordecone [8] and acrolein [9]. Similarly, aldo–keto reductase of rat liver metabolizes aflatoxin secreted by *Aspergillus flavus* [10].

In humans, AR activity is associated with diabetic complications such as hyperglycemia [1,4,11]. In mammalian renal medullary cells, AR activity appears to be associated with osmoregulation [11]. AR is considered as a key enzyme of the polyol pathway leading to the accumulation of sorbitol [12] in stress situations, which is associated with
maintenance of osmotic balance of the cytoplasm and protection of the function of macromolecules in both animals and plant systems [12,13].

In plants, not much is known about AR, particularly their role in stress response processes. AR protein was reported as one of the desiccation responsive-proteins of barley embryos showing sequence similarities with that of human AR, frog eye lens rho crystallin and bovine prostaglandins [2]. In barley, AR accumulates during a specific stage of embryogenesis where embryos acquire desiccation tolerance [2]. It was the first report suggesting a functional role for this protein in the desiccation tolerance processes of embryos. Studies on AR activity in other crop plants are sporadic. Of the tested Gramineae members, only wild oats [6] and bromegrass [14] were reported to accumulate AR protein. Further AR accumulation was also reported in desiccated leaves of an African resurrection plant, Craterostigma plantagineum suggesting an osmoprotective function [2].

The present paper deals with the identification of AR protein and enzyme in mature seeds of rice and some members of Gramineae. We report that this protein/enzyme is induced in vegetative tissues of rice lines by water stress, salt stress and exogenous ABA application, and its inducibility is genotype dependent. We further show that AR is developmentally regulated in rice.

2. Methods

2.1. Plant material

Rice lines used in the present study include both cultivars and land races. Annada, Hamsa, and Tulasi are well known cultivars belonging to indica subspecies which were originally obtained from DRR, Hyderabad, India. Hamsa is a known drought sensitive line while other lines exhibit a varying degree of tolerance. K39 is a cold tolerant cultivar grown in the North Eastern regions of India. The plants were grown in the green house or field for several generations. Other cereals used in the study include Zea mays, Avena sativa, Eleucine coracana, Hordeum vulgare, Triticum aestivum, Sorghum bicolor and Pennisitum typhoidieum.

2.2. Plant growth and stress treatment

Seeds were surfaced sterilized with 5% sodium hypochlorite (NaOCl) for 5 min and rinsed with distilled water several times. They were grown on rough filter papers in see-through plastic germination boxes in growth chambers with 12 h light/dark cycle at 28 ± 2°C. The 8 day old seedlings were treated with 20% polyethylene glycol (PEG 6000), ABA (100 μM), NaCl (200 mM) or kept in the cold at 5 ± 1°C for a specific number of days. Control plants were grown normally without any stress treatment. The seedlings were harvested at regular intervals and quick frozen in liquid nitrogen or immediately processed.

2.3. Total soluble protein extraction

Leaf and developing seed (at different days after pollination, DAP) from young seedlings and panicles respectively, were collected, quickly frozen in liquid nitrogen and used for protein extraction. The total proteins from embryos, immature and mature seeds were isolated according to Rao et al. [15] with modifications. Samples were finely ground in liquid nitrogen and extracted with hot (95°C) buffer (1gm/2 ml) containing 0.25M Tris–HCl (pH 8.0), 0.4% SDS, 20 mM EDTA, 2 mM PMSF and 5% β-mercaptoethanol, centrifuged at 12 000 × g for 10 min, and proteins in the supernatant were precipitated with 15% TCA at 4°C. The pellet was washed three times with 80% acetone and dissolved in Tris buffer (pH 8.0). The protein concentrations were determined by the Bradford method [16].

2.4. Partial purification of aldose reductase

Partial purification of AR was performed according to the protocol described in Bartels et al. [2] with minor modifications. One gram of fresh shoot tissue, mature seeds and embryos were finely ground in liquid nitrogen and extracted with 10 ml of extraction buffer containing 20 mM potassium phosphate buffer, pH 7.0, 5 mM β-mercaptoethanol and 0.5 mM EDTA. The homogenate was spun at 12 000 × g for 20 min and the supernatant was collected and saturated with 40% ammonium sulphate. The supernatant was further raised to 80% ammonium sulphate saturation. The proteins thus precipitated were dissolved and di-
analysed over night at 4°C in the same extraction buffer. Aliquots of the fractions were used for both enzyme activity and Western analysis.

2.5. **Determination of AR activity**

AR activity was spectrophotometrically determined by measuring the decrease in the concentration of NADPH at 340 nm for 5 min at room temperature [2]. The assay mixture contained 100 mM sodium phosphate buffer, pH 6.9 and 0.15 mM NADPH with 25 mM DL-glyceraldehyde as substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per min under the conditions mentioned above. Specific activity was expressed as units per mg protein.

2.6. **SDS-PAGE and Western analysis**

SDS-PAGE was performed according to Laemmli et al. [17]. Fifty–100 μg of extracted proteins of control and treated samples were fractionated on 15% SDS-PAGE at 150V and then electrophoretically blotted onto nitrocellulose paper at a constant voltage of 30V at 4°C [18]. The membranes were stained with Ponceau-S solution and the molecular weight standards were marked. The blots were probed with the primary antibody (pG22-69) raised against the barley desiccation-responsive AR protein from embryo [2]. The secondary antibody, HRPO goat anti-rabbit IgG conjugate (Boehringer Mannheim) was used according to the manufacturer’s specifications. The blots were incubated in the presence of the substrate, 4-chloro-1-naphthol and hydrogen peroxide and the complex was visualized by the development of purple color.

3. **Results**

3.1. **Identification of aldose reductase-related protein in rice seeds**

Rice genotypes were analyzed for the presence of AR like protein in both seeds and vegetative tissue. The Western blot data clearly showed that antibodies raised against the desiccation-responsive AR protein from barley embryo detected a 34-kDa protein in the mature seeds of Hamsa and K39 (Fig. 1). On the contrary, this protein was not detected in shoots and roots of the seedlings grown under normal conditions. The AR activity in partially purified extracts from seeds was monitored using DL-glyceraldehyde as substrate in the presence of NADPH as a co-factor. There was negligible activity when fructose and glucose were used as substrates (data not shown). Earlier reports indicated that purified aldose reductase from acidophilic and thermophilic red alga showed a high affinity towards DL-glyceraldehyde followed by xylose [19].

3.2. **Developmental profiles of AR-related protein during embryogenesis and germination**

AR protein accumulation during embryogenesis was analyzed. Proteins isolated from immature seeds of K39 at different stages of maturation were fractionated on SDS-PAGE and the blots were probed with anti-AR antibodies. Western analysis revealed a stage specific accumulation of this protein, reminiscent of the typical LEA pattern [20,21]. AR-like protein begins to appear at 15 DAP and increases as the embryogenesis pro-

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**Fig. 1. Immunodetection of the AR-related protein in rice:** The proteins from different plant parts were separated on SDS-PAGE and blotted onto nitrocellulose filter paper and probed with pG22-69 antibodies raised against barley embryo protein. HRPO anti IgG conjugate was visualized by purple color precipitable substrate in the presence of 4-chloro-1-naphthol and hydrogen peroxide. The standard molecular weight markers are shown on the left. On the right side of the panel the position of the 34-kDa aldose reductase-related protein is shown by an arrowhead. Twenty microgram protein was loaded onto each lane.
Induction of AR protein in rice shoots by ABA and other abiotic stresses

The effects of abiotic stress on AR protein and enzyme levels were investigated. Activity assays revealed a significant increase in AR activity in shoots exposed to ABA, PEG and NaCl treatments (Fig. 3a). The increase was maximum in response to ABA (53% increase) followed by PEG (40%) and NaCl (35%).

Western analysis revealed that this protein was induced in shoots of K39 seedlings treated with 100 μM of ABA, water stress (PEG 20%) and salinity (200 mM) stress (Fig. 3b). The protein fraction utilized for the immunoblotting and enzyme analysis was the same. Moreover, quantitative induction of this protein in response to ABA and abiotic stresses was nearly proportional to the increase in the enzyme activity. However, there was no change in AR levels under cold stress (Fig. 3a, b).

AR protein and enzyme activity in seeds and embryos are relatively higher than that in stressed vegetative tissue (Fig. 3a, b). Further, embryo showed significantly more AR enzyme activity (35% more) than seed.

Variatel differences in the ABA-inducibility of AR-related protein

There were clear differences in the ABA inducibility of AR-related protein among the rice varieties deploying different levels of drought tolerance. Application of 100 μM of ABA was found to induce this protein in drought tolerant varieties, Annada and Tulasi, but not in the sensitive cultivar, Hamsa, as revealed by Western data (Fig. 4a) and enzyme activity analysis (Fig. 4b). Interestingly, while Annada showed a detectable increase in enzyme levels 3 days after ABA treatment, Tulasi was conspicuously lacking such an increase. However, at 5 days after ABA treatment, both Annada and Tulasi showed 58 and 49% increases in enzyme levels, respectively (Fig. 4b).

AR accumulation is evolutionarily conserved

In view of the relevance of AR to biosynthesis of sorbitol, which has been reported in many higher plants [13], we have investigated the existence of the AR-related protein in mature seeds of other cereals. SDS gels run from crude protein extracts reaching the maximum at maturation (Fig. 2a). Upon germination, the AR protein levels gradually decrease and reach a minimum by the 5th day after germination (DAG) (Fig. 2b).
extracts of mature seeds of several cereals such as rice, barley, *Sorghum*, *Eleucine*, pearl millet, oat, wheat and maize were blotted and probed with the same Anti-AR antibodies. A single protein of molecular weight of 34-kDa was detected in mature seeds of all the tested members (Fig. 5a). Interestingly, this protein in rice, barley, *Sorghum*, *Eleucine*, pearl millet, maize and wheat appears to be of similar apparent molecular weight. However in oats, AR-related protein seems to be slightly smaller than the others. Significantly this protein was not detectable in the leaf tissue of any of these plants. Interestingly, AR protein is detected in ABA treated leaf tissues of rice seedlings but not in other cereals (Fig. 5b).

4. Discussion

Our results highlight the identification of AR in mature seeds of rice and other members of Gramineae. Further, the results demonstrate that the aldose reductase accumulation in rice is stress responsive and varietal specific. The AR begins to accumulate from 15 DAP and reaches maximum accumulation at mature seed stage as revealed both by the Western and enzyme activity analyses (Fig. 2a and Fig. 3a, b). Such a developmental pattern of aldose reductase accumulation closely resembles that of endogenous ABA levels in maturing seeds of rice [22]. Further, the developmental pattern of AR accumulation during late embryogenesis in rice resembles that of LEA proteins which are widely studied and implicated in desiccation tolerance in late embryogenesis in a variety of plant species [20,21]. LEA proteins have been implicated in conferring protection to cellular organelles through different ways based on their conserved domains [23]. LEA proteins are predicted to have an enhanced water binding capacity, act like molecular chaperones, and some are predicted to play a role in the sequestration of ions that are concentrated during cellular dehydration [20,24]. In wheat seedlings during dehydration, a correlation was reported between organ survival and group 3 LEA protein accumulation [25].

Our studies on AR accumulation in late embryogenesis further support the probable utility of proteins with LEA characteristics in conferring stress tolerance during this desiccation phase. Particularly, the identification of a stress/ABA responsive enzyme involved in biosynthesis of an osmolyte (in this case sorbitol) with LEA characteristics is an indication of employing the con-
Fig. 4. (a) Western blot showing the differential accumulation of AR-related protein in rice cultivars by ABA application: Proteins from different rice cultivars treated with ABA were separated on SDS-PAGE and blotted onto nitrocellulose filter paper and probed with pG22-69 antibodies. The standard molecular weight markers are shown on the left. The arrowhead points to the position of the 34-kDa aldose reductase-related protein. Thirty microgram protein was loaded onto each lane. (b) ABA-induced changes in aldose reductase activity in seedlings of different cultivars. Rice seedlings were treated with ABA (100 μM) for 3 days and 5 days. The enzymatic activity represented in μmoles of NADPH oxidized per min per mg of protein. Each value represents mean ± S.D of three replicates.

Our observations therefore provide the clue to the presence of AR in embryos of maturing seeds of rice, and its possible role in desiccation tolerance processes. AR association in rice with the desiccation phase of seed maturation and its disappearance on germination is similar to the pattern of AR transcript accumulation and decline during seed maturation and germination respectively, in barley and wild oats. [2,6].

4.1. AR is responsive to exogenous ABA and other abiotic stresses

AR protein is induced in the shoots of some rice genotypes by the exogenous application of ABA, water stress and salinity, but not by cold stress. Western data are in agreement with increased enzyme activity levels. The increase was maximum in response to ABA (53% increase) followed by PEG (40% increase) and NaCl (35% increase). However, under cold acclimation, there was no significant increase either in protein or enzyme activity levels leading to the conclusion that AR is primarily responsive to osmotic stress. The presence of substantial AR enzyme activity but not immunodetectable AR protein (Fig. 3a as compared to Fig. 3b and Fig. 4a as compared to Fig. 4b) indicates the presence of other enzymes (proteins) having AR activity that are not recognized by the AR antibodies. Furthermore, both seeds and embryos showed higher levels of enzyme activity and protein levels (Fig. 3a, b) than vegetative tissue. Among all the tested tissues, embryos accumulate highest levels of AR protein and enzyme activity suggesting that AR is primarily synthesized in embryos under normal conditions and induced in other tissues under stress conditions.

As AR protein accumulation in vegetative tissues is primarily associated with osmotic stress, it is not clear whether increased endogenous ABA is the cause for such an accumulation. Bartels et al. [2] showed that AR accumulation in desiccated leaves of Craterostigma, providing further evidence that it might play a role in osmoprotection. It is known that a majority of the LEA proteins from various crop plants are induced in response to ABA and abiotic stress conditions in vegetative tissues [20,21]. In bromegrass cell suspensions, ABA treatment induced significant levels of an AR mRNA and enzyme activity levels [14]. In wild oats, water stress leads to the induction of the AR transcript [6].

observed regulatory mechanisms for stress tolerance at particular stages of plant development that are vulnerable to desiccation damage. The mode and means of LEA-type proteins in conferring protection are obviously different, varying from being structural protectors such as chaperones [26] to the enzymes involved in osmolyte biosynthesis. Earlier studies with barley and wild oats further suggest the association of aldose reductase with desiccation tolerance of maturing embryos [2,6,27,28].
4.2. AR accumulation in vegetative tissue under stress is genotype dependent

An important observation in the present study is the absence of AR in ABA-treated shoots of a drought sensitive cultivar, Hamsa (Fig. 4a, b). All the other tested cultivars, which are relatively drought tolerant, accumulate this protein under stress. This could be due to the genetic background differences that exist between the varieties. We propose that AR protein in rice is associated with desiccation tolerance processes both in seedlings as well as embryos. It is tempting to speculate that Hamsa plants are genetically deficient in a regulatory element that governs the expression of AR, particularly in vegetative tissues under stress or in response to ABA. It is to be noted that Hamsa seeds do accumulate AR as a normal developmental cue. Numerous earlier reports showed that stress induced proteins accumulate both in sensitive and tolerant cultivars, though there are quantitative differences [29,30]. Our results, however, clearly show that the tolerant lines selectively accumulate aldose reductase under stress. Our observations will be of some use in genetic characterization of the AR locus and its role in drought tolerance in rice. However, further studies with AR are needed to unequivocally associate AR with stress tolerance processes and use it as a potential marker for the stress tolerance phenotype.

4.3. Occurrence in Gramineae members

Our results clearly demonstrate the occurrence of AR protein in mature seeds of other cereals, such as maize, barley, wheat, Sorghum, Eleucine, pearl millet and oats. With the exception of oats, all cereals tested show AR of similar apparent molecular weight. Li and Foly [6] reported that the AR clone (pAF30) from wild oats showed 90% homology in predicted amino acid sequence with respect to the putative AR gene from barley [2] and bromegrass suspension cells [14].

The stress responsive AR induction in vegetative tissues reveals that there are significant differences between cereals. Of all the cereals tested, only rice shows the induction of AR under ABA, water stress and salinity. In wild oats the AR gene is found to be ABA responsive in embryos and is inducible under water stress in seedlings [6]. In mammalian renal medullary cells, an increase in role in drought tolerance in rice. However, further studies with AR are needed to unequivocally associate AR with stress tolerance processes and use it as a potential marker for the stress tolerance phenotype.
AR activity was detected under salinity stress [1,11]. In response to osmotic stress, renal cells accumulate the osmolyte sorbitol through increased transcription of the AR gene and its osmotic response element (ORE) has been identified [31]. These results further suggest that the osmoregulatory processes were evolutionarily conserved.

The accumulation of different osmolytes in cytoplasm in response to various abiotic stress conditions is a well-established phenomenon in plants [32]. Among the osmolytes, proline, betaine and sugar alcohols have been implicated in osmoregulatory function in plants and have been thought to be of great adaptive value [33]. The identification of AR in rice and other cereals assumes importance in view of its role in the synthesis of the sugar alcohol, sorbitol. It plays an important role in osmotic adjustment in apple leaves during water stress [34], was also found in corn [35], Rosaceae members [36,37] and soybean [38]. Salt tolerant Plantago species accumulate significant levels of sorbitol [39]. In apples, sorbitol biosynthesis during water stress is mediated by an enzyme aldose-6-phosphate reductase [34].

The transgenic approach seems to be the preferred one for the elucidation of the role of AR in stress response pathways. The over production of osmolytes, proline, mannitol and ononitol in cytoplasm of transgenic tobacco plants was proved to confer stress tolerance [40–42]. With the availability of reproducible and routine rice transformation techniques, it would soon be possible to test the role of AR in stress tolerance. The elaboration of the genetic and biochemical basis of osmolyte pathways, including the polyol pathway and the characterization of the regulatory elements that govern the expression of AR would help in the genetic improvement of the stress response in rice.

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