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Association between accumulation of allene oxide synthase activity and development of resistance against downy mildew disease of pearl millet

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Abstract The present study was aimed at understanding the possible association of allene oxide synthase (AOS), an enzyme implicated in the octadecanoid pathway during the pearl millet-downy mildew interaction. AOS 13-HPOT (13-hydroperoxy-9,11,15-octadecatrienoic acid) metabolizing activity assays assessed in various pearl millet cultivars with differential resistances against downy mildew revealed a positive correlation between cultivar resistance levels and AOS activities. Furthermore, the involvement of AOS in response to downy mildew was demonstrated by induction of AOS activity in both susceptible and resistant pearl millet cultivars during *Sclerospora graminicola* infection with higher induction observed in the resistant cultivar. Consistently, western blot analysis and tissue-blot immunoassay demonstrated the remarkable increase in AOS protein accumulation in the incompatible interaction. In addition, the tissue-blot immunoassay also showed the compartmentalization of AOS in the epidermis and vascular bundles of pearl millet seedlings. Expression analysis of a putative *PgAOS1* gene revealed a marked difference in accumulation of *PgAOS1* transcripts between contrasting plants, with pathogen-induced higher accumulation of the transcripts observed only in the resistant cultivar; a result which is in agreement with pathogen-induced AOS level and activity, indicating that *PgAOS1* plays an important role in regulation of AOS level and activity in pearl millet upon *S. graminicola* infection. Our findings suggest an important role for AOS in regulation of responses to downy mildew disease in pearl millet. The differential AOS activities can potentially be used for selection of new disease-resistant pearl millet varieties, and the identified AOS-encoding gene(s) as genetic resource for development of enhanced downy mildew-resistant cultivars.

Keywords Pearl millet · Downy mildew resistance · Allene oxide synthase activity · Transcript accumulation · Tissue-blot immunoassay · Western blot analysis

Introduction

Plants, when being challenged by pathogens, herbivores or mechanical stresses, initiate appropriate defense reactions to control the crisis. The octadecanoid pathway plays a very important role in plant defense mechanisms against insect and pathogen attacks [1, 2]. The resulting host responses significantly contribute to the defense status of the plants by increasing the amount of several defense proteins and phytochemicals that collectively abolish or retard the aggressors' invasion [3, 4]. Allene oxide synthase [AOS (EC 4.2.1.92)] is an intermediary enzyme in the octadecanoid pathway involved in conversion of (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) to allene oxide [5, 6].

Plant AOSs are described as non-classical cytochrome P450 enzymes [7]. Most of them belong to the CYP74A subfamily as they do not require molecular oxygen or

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reducing equivalents from NADPH for their activity. AOS is known as the target point in the regulation of Jasmonic acid (JA) in defense responses [8]. Several full-length AOS encoding cDNAs have been isolated and characterized from various dicot plants, such as flax [9], pea [10], guayule [11], *Arabidopsis* [12], tomato [13, 14], soybean [5] and passion fruit [15], and from monocots, such as barley [16] and rice [17]. In flax, a putative *AOS* gene was reported to be expressed constitutively in different organs. However, mechanical wounding was shown to enhance the expression of this *AOS* gene, both locally at the site of wound and systemically at distant healthy leaves [18]. In pea, expression of a putative *AOS* gene was demonstrated to be induced by treatment with *Mycosphaerella pinodes* [10]. The expression analysis of the *HnAOS* gene in *Hyoscyamus niger* indicated that *HnAOS* expression was significantly induced by methyl jasmonate (MeJA) and wounding stress [19]. Consistent with this finding, the AOS-spectrophotometric assays showed that 13-HPOT was metabolized faster in passion fruit extracts from MeJa-treated plants when compared with extracts from the non-treated control. Furthermore, western blot analysis confirmed that the MeJA treatment resulted in strong accumulation of AOS in passion fruit over the control [15]. Conversely, transgenic rice plants overexpressing the pathogen-inducible *OsAOS2* gene showed higher accumulation of JA level, upregulated expression of pathogenesis-related genes and increased resistance to *Magnaporthe grisea* infection [20].

Pearl millet (*Pennisetum glaucum*) is currently the sixth most important cereal crop grown in the arid and semi-arid regions of the world. Pearl millet occupies 50 % of the total millets grown in the world, and India produces more than half the amount in an area of ten million hectares [21]. A major biotic constraint in pearl millet production is the downy mildew disease, which is caused by *Sclerospora graminicola*, an oomycete pathogen. *Sclerospora graminicola* is highly destructive and widespread, resulting in yield loss estimated up to 80 % [21]. Although our earlier studies have suggested the involvement of various enzymes, such as, β-1,3-glucanase [22], peroxidase [23], proton ATPase [24], superoxide dismutase [25], phenylalanine ammonia lyase [26], lipoxygenase [27] and polyphenol oxidases [28] in the resistant interaction of pearl millet against *S. graminicola*, the possible role of AOS in enhancement of the pearl millet resistance against downy mildew disease remains elusive.

Hence, in the present study we aim to investigate the involvement of AOS during pearl millet-downy mildew interaction by comparative analysis of contrasting pearl millet cultivars. Specifically, AOS enzyme activity, immuno-histochemical accumulation of AOS protein and expression of *AOS* gene were compared in the resistant and susceptible pearl millet cultivars during *S. graminicola* treatment to provide evidence for the positive correlation between AOS and resistance against downy mildew.

Materials and methods

Plant material and chemicals

Ten pearl millet cultivars (IP18292, ICMP451-P6, ICMR-01004, IP18293, H77/833-2, 700651, P310-17, 7042S, 843B and 852B), representing diverse geographical origins, were collected and used as potential differential or contrasting cultivars in response to downy mildew disease in the present study. The genotype seeds were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India through All India Co-ordinated Pearl Millet Improvement Project (AI-CPMIP), Mandor, India. Linolenic acid and soybean lipoxygenase were purchased from Sigma-Aldrich Pvt. Ltd. (Bangalore, India).

Screening of plants for downy mildew disease incidence

The disease responses of potentially contrasting genotypes were analyzed by field screening for downy mildew disease for classification of the collected genotypes into four categories (highly resistant, resistant, susceptible and highly susceptible). The screening and scoring of the disease were performed as described earlier [29] at the experimental station of Indian Council of Agricultural Research (ICAR) downy mildew sick plot of the Department of Studies in Biotechnology, University of Mysore, Mysore (N26°18′, E73°30′, 817 m altitude, red loam soil), India during kharif seasons 2007–2009.

Pathogen and inoculum preparations

Sclerospora graminicola, the downy mildew pathogen of pearl millet, was isolated from its susceptible cultivar (7042S) and maintained under greenhouse conditions at 22 ± 2 °C with 90 % relative humidity. Infected leaves were collected in the evening and washed under running water to remove the remnants of the earlier sporulation and the leaf surfaces were then blot-dried. The leaves were placed on petri dishes lined by moist blotters and incubated under humidity chambers overnight. The sporangia were harvested next morning into sterile distilled water for the release of zoospore. The concentration of the zoospore in the suspension was adjusted to 4×10^4 zoospores/ml with sterile distilled water using haemocytometer [30].

Inoculation of seedlings and sampling

The pearl millet universal highly resistant cultivar IP18293 and highly susceptible cultivar 7042S seeds were germinated in perplex petri dishes lined with the moist blotter discs at 25 ± 2 °C for 2 days. The root-dip inoculation of the 2-day-old plants with 4×10^4/ml was carried out according to the protocol detailed earlier [30]. The seedlings were then harvested at 0, 3, 6, 9, 12, 24, 48 and 72 h and stored at −20 °C for subsequent experiments. Uninoculated distilled water-treated seedlings served as control.

AOS activity assay

Enzymes were extracted from all the harvested samples according to the method reported earlier [15]. Two grams of powdered pearl millet seedlings were homogenised with 3 ml ice-cold extraction buffer [50 mM potassium phosphate, pH 7.5 and 10 % (w/w)] insoluble polyvinylpyrrolidone. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged for 30 min at 13,000×*g* at 4 °C. The protein content of the extracts was determined according to the standard procedure [31] using bovine serum albumin (Sigma, USA) as a standard according to manufacture's instructions (BioRad).

Preparation of fatty acid hydroperoxide

13-HPOT to be used as substrate for the AOS assay was prepared as previously described [32] using soybean lipoxygenase and linolenic acid.

Spectrophotometric assay for AOS

The AOS 13-HPOT-metabolizing activity in various pearl millet seedlings was measured spectrophotometrically by monitoring the rate of decrease in absorbance at 234 nm [11, 33]. The enzymatic assays were performed at 25 °C in 1 ml of 50 mM phosphate buffer, pH 7 containing amount of 20 µl of 13S-hydroperoxylinolenic acid (stock solution: 1 mg/ml in ethanol) equivalent to an absorbance of 1.5 at 234 nm. The absorbance was recorded for 2 min following the addition of 50 µg total protein using a Hitachi (U 2000, Tokyo, Japan) UV–visible spectrophotometer. The specific activity was expressed as a change in the absorbance at 234 nm/mg protein/min.

Electrophoresis and western blotting

The total protein was extracted from both uninoculated and pathogen-inoculated resistant (IP18292) and susceptible (7042S) seedlings harvested at 24 h post inoculation. Proteins were separated on a 12 % gel by SDS-PAGE with concentrations of 50 µg/lane [34]. The separated proteins were blotted on to polyvinylidene di-fluoride (PVDF) membrane using multiphor II (Pharmacia, Freiburg, Germany) electrophoretic transfer apparatus according to the manufacturer's protocol. The blot was blocked in 2 % skimmed milk Tris buffered saline (TBS: 10 mM Tris–HCl, pH 8.0 containing 150 mM NaCl). Subsequently, the blot was probed with rabbit anti-AOS antibody (1:1000 dilution in blocking buffer; a kind gift from Wasternack C, Institute of Plant Biochemistry, Halle, Germany) and developed according to previously mentioned protocol [35].

Tissue-blot immunoassay

Tissue-blot immunoassay was carried out for inoculated and uninoculated resistant and susceptible treated seedlings harvested at 24 h following the method of Whitfield et al. [36]. The seedlings were sliced at the region of coleoptile and the cut ends were blotted on to 0.45-µm NITRO ME nitrocellulose membranes (Micron separations Inc., Westboro, MA) applied with consistent normal thumb pressure for 3–5 s. The blotted membranes were developed according to the protocol mentioned earlier [35]. Blots were observed under stereo binocular microscope (Leica MS5, Germany) with high magnification (100×). The images were captured with the digital camera attached to the microscope using the RS Image Analysis Software (Roper Scientific Photometrics, Tuscon, AZ).

Cloning of the pearl millet AOS probe fragment

Total RNA was extracted from pearl millet IP18292 resistant seedlings using the phenol–chloroform method described previously [37]. The first strand cDNA was prepared using 2-step AMV (Avian Myeloblastosis Leukemia Virus) RT-PCR Kit (Qiagen, Bangalore) as per the kit's instructions. The oligonucleotide primers were designed based on other known plant AOS protein sequences. The protein sequences were aligned into blocks using Blockmaker (http://www.bloacks.fhcrc.org/blocks/make_blocks.html). The block with the best alignment was further subjected to the programme Codehop (http://www.blocks.fhcrc.org/codehop.html) to design the degenerate primers AOSF1-CCGGGACGAGGCCTGYCAYAAYYT and AOSR1-GTTCTTGCCGGGGCAYTGYTTRTT. The oligonucleotides synthesized by Sigma-Aldrich Pvt. Ltd., Bangalore were used for the PCR amplification. The 512-bp PCR product was cloned into pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit (Fermentas Life Sciences) and transformed into *E. coli* JM109 competent cells using the TransformAid™ Bacterial Transformation Kit (Fermentas Life Sciences). The plasmids from the

positive clones were sequenced at MWG Biotech Pvt. Ltd., Bangalore, India.

Bioinformatics analysis

The identity of the 512 bp sequence obtained above was obtained by BLAST search analysis at NCBI. Phylogenetic analysis of the pearl millet AOS CypX domain and other known monocot and dicot AOS CypX domain sequences obtained from the NCBI nucleotide database was performed using the bioinformatic tools available at http://www.phylogeny.fr [38]. The consensus sequences (including CypX domain region) of pearl millet AOS and other known monocot and dicot AOSs obtained from NCBI database were aligned using MUSCLE, curated using G-blocks tool and then submitted to PhyML for phylogenetic analysis and the tree was rendered using TreeDyn. The phylogenetic analysis used ALTR statistic test for tree computation which is very fast unlike the bootstrap analysis. The branch support values generated by the ALTR test is an equivalent of the bootstrap values of the bootstrap analysis. The multiple alignments of AOS protein sequences were carried out using the BLOSUM62 scoring matrix with pearl millet AOS sequence as the reference (Clone Manager 9 Professional Edition).

Northern blotting and hybridization analysis

Total RNA was extracted from harvested pearl millet seedlings with and without *S. graminicola* by the phenol–chloroform method as described previously [37]. RNA (10 μg) was denatured, separated by electrophoresis, transferred to Hybond-N1 membrane (Amersham Pharmacia, Piscataway, USA) in 20× saline sodium citrate (SSC) (3 M NaCl, 0.3 M sodium citrate), and fixed onto the membrane by baking at 80 °C for 90 min. RNA gel blots were pre-hybridized in a solution containing 0.25 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7 % sodium dodecyl sulfate (SDS, w/v), and 1 mM ethylenediaminetetraacetic acid at 60 °C for 3 h. The blots were hybridized with 5 μCi α^{32}P-labeled 512 bp *PgAOS1*as probe in the same solution overnight at 60 °C. The membranes were washed twice for 20 min each at 60 °C in 0.2 % SSC and 0.1 % SDS (w/v). The hybridized blots were exposed to Phosphorimager plates appropriately and scanned with Multifunctional Image Analysis System (FLA 5000; FujiFilm, Tokyo, Japan).

Statistical analysis

For the field experiments, the data from each experiment consisted of three replicates of 27 plants/genotype were subjected to analysis of variance (ANOVA) using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA). Significant effects of genotypes were determined from the magnitude of the F-value ($P < 0.05$). Values are expressed as mean ± standard error. Treatment means were separated using Tukey's HSD (honest significant differences) test.

For comparison of AOS activities in pearl millet susceptible (7042S) and resistant (IP18292) seedlings with or without *S. graminicola* treatment, two independent experiments with three biological repeats/each experiment were performed. A Student's *t* test was then used to determine significant differences in AOS activities (n = 6) between inoculated and uninoculated samples at each given time point.

Table 1 Allene oxide synthase (AOS) activity in different host differential genotypes of pearl millet measured after 24 h post-inoculation with downy mildew pathogen *S. graminicola*

Genotypes	Mean downy mildew incidence in field	Category based on field screening in Kharif (2009–2011)	AOS activity (change in absorbance at 234 nm/mg protein/min)
7042S	97^{a}	Highly susceptible	3.5 ± 0.34^{e}
843B	38^{c}	Highly susceptible	6.2 ± 0.54^{d}
852B	66^{b}	Highly susceptible	4.7 ± 0.19^{de}
700651	24^{d}	Susceptible	8.1 ± 0.22^{c}
P310-17	16^{de}	Susceptible	10.6 ± 0.37^{c}
ICMR01004	05^{f}	Highly resistant	26.4 ± 0.17^{ab}
ICMP451-P6	09^{f}	Resistant	21.2 ± 0.47^{b}
IP18292	07^{f}	Resistant	24.4 ± 0.31^{b}
IP18293	04^{f}	Highly resistant	28.9 ± 0.25^{a}
H77/833-2	03^{f}	Highly resistant	30.7 ± 0.47^{a}

Data presented are mean ± SE of three independent experiments. Different superscript letters (a, b, c, d, e and f) within a column indicate a significant difference from each other in all combinations (Tukey's HSD (honest significant differences) test, $P < 0.05$)

Results

The resistance degree of pearl millet cultivars to downy mildew disease is positively correlated with AOS activity

Various pearl millet cultivars, which possess different levels of resistance or susceptibility to downy mildew disease, were collected from different regions of India to study whether the resistance to downy mildew disease is associated with AOS levels. Based on the field screening results shown in Table 1, the obtained ten cultivars were classified into four categories, from highly resistance to highly susceptibility: highly resistant genotypes (H77/833-2, IP18293 and ICMR-01004), resistant genotypes (IP18292 and ICMP451-P6),

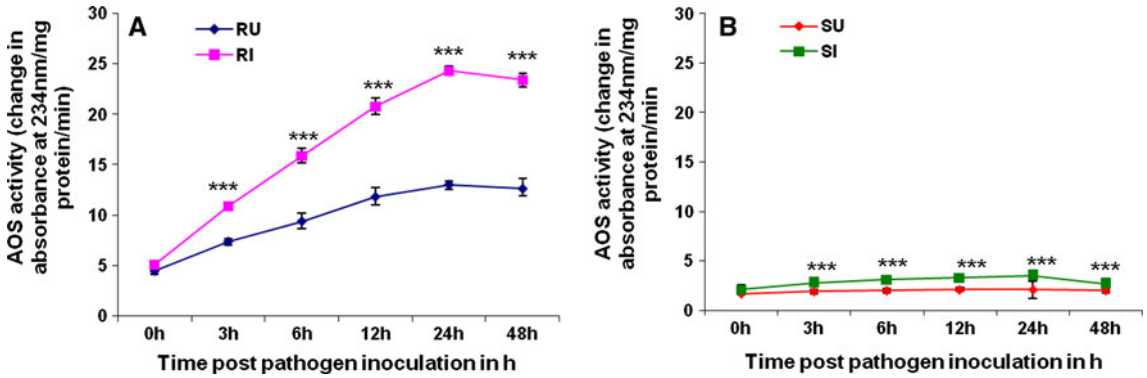

Fig. 1 Time course expression pattern of 13-HPOT-metabolizing activity. **a** 13-HPOT-metabolizing activity in IP18292 resistant cultivar treated with water (RU, control) or *S. graminicola* (RI) for indicated time points. **b** 13-HPOT-metabolizing activity in 7042S susceptible cultivar treated with water (SU, control) or *S. graminicola* (SI) for indicated time points. Data presented are mean of six biological repeats from two independent experiments (3 repeats/experiment). *Bars* indicate the standard errors. *Asterisks* indicate significant differences as determined by a Student's *t* test (***$P < 0.001$)

susceptible (700651 and P310-17) and highly susceptible genotypes (7042S, 843B and 852B).

Next, we assessed the AOS levels in all the pearl millet cultivars 24 h post-inoculation with *S. graminicola* by analyzing the 13-HPOT-metabolizing activities of the AOS in the extracts. All the highly resistant and resistant genotypes displayed significantly higher AOS activity, ranging from 21.2 to 30.7/mg protein/min over the highly susceptible and susceptible genotypes showing from 3.5 to 10.6/mg protein/min. This result indicated that there is a positive correlation between the AOS levels and downy mildew disease incidence under field conditions (Table 1), suggesting that AOS might contribute to enhanced resistance against downy mildew disease.

Comparative analysis of AOS activities in resistant and susceptible pearl millet cultivars with and without pathogen inoculation

As a means to determine that the AOS may play a role in response to *S. graminicola* infection in pearl millet, we individually examined the AOS activities temporally in the extracts of pathogen-inoculated and -uninoculated IP18292 resistant and 7042S susceptible cultivars and compared their AOS activities. Spectrophotometric assay showed that 13-HPOT was metabolized much faster in protein extracts of inoculated resistant (RI) seedlings when compared with the uninoculated seedlings of the same cultivar (RU). In RI seedlings the AOS activity gradually increased with the highest activity of 24.3/mg protein/min recorded at 24 h, which was approximately 1.9-fold of that in RU line (Fig. 1a). AOS activity was much lower in the uninoculated susceptible (SU) cultivar in comparison with RU line as shown in Fig. 1a, b. Furthermore, although a pathogen-induced increase in AOS activity was noted when the susceptible variety was treated with *S. graminicola*

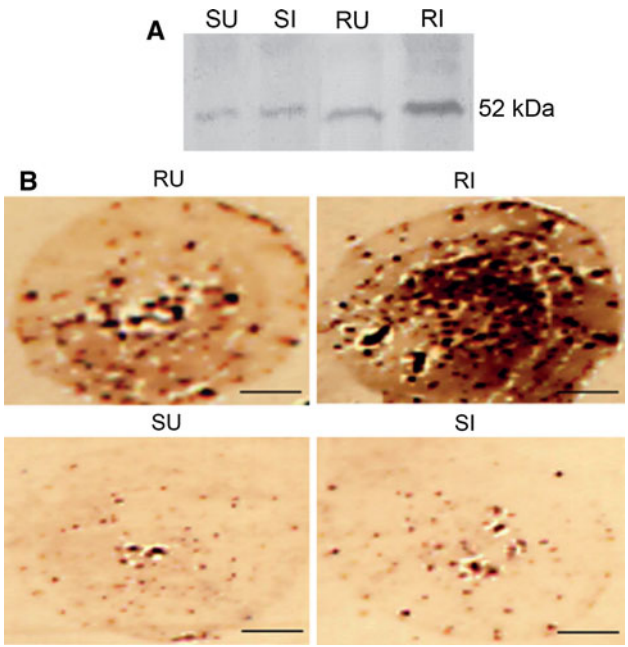

Fig. 2 Comparative analysis of AOS protein expression in resistant and susceptible cultivars. **a** Western blot analysis of AOS protein in IP18292 resistant and 7042S susceptible cultivars treated with water (RU and SU, controls) or *S. graminicola* (RI and SI) for 24 h. Each lane was loaded with 50 μg of the total protein extract. Anti-AOS polyclonal antibody generated in rabbit against barley AOS was used for the analysis. **b** Tissue-blot immunoassay of AOS protein in IP18292 resistant and 7042S susceptible cultivars treated with water (RU and SU, controls) or *S. graminicola* (RI and SI) for 24 h. *White arrows* indicate the accumulation of AOS on the vascular bundles (cortex region) and the presence of AOS in epidermis is shown in *black arrows*. Tissue-blot immunoassay of the cross-sections of coleoptile regions from pearl millet samples were obtained by printing them onto nitrocellulose membrane. Anti-AOS polyclonal antibody generated against barley AOS was used for the analysis (*Bar* = 200 μM)

[inoculated susceptible (SI) cultivar], the activity levels were much lower in comparison to those observed in the resistant samples (Fig. 1b). A significant pathogen-induced

increase in AOS activity observed only in the resistant cultivar suggests the involvement of AOS in host response to *S. graminicola*.

Comparative analysis of AOS enzyme levels in resistant and susceptible pearl millet cultivars with and without pathogen inoculation

We were then interested in whether the increase in AOS activity in response to *S. graminicola* infection was correlated with the AOS levels. Thus, we carried out a western blot analysis using immune serum raised against barley AOS. Our result revealed that the IP18292 RI cultivar displayed an intense accumulation of an immuno-reactive protein band of 52 kDa after 24 h of inoculation with *S. graminicola* over the RU control (Fig. 2a). In comparison with the RU cultivar, both SI and SU seedlings showed a faint band of 52 kDa with similar intensity (Fig. 2a). The western blot and AOS activity assay results in both resistant and susceptible cultivars, following 24 h of infection, indicated a good correlation between the protein abundance and the corresponding AOS activity levels (Figs. 1, 2a).

Consistent with the data of western blot analysis, microscopic observations of the tissue-blot immunoassay indicated that the abundance of AOS was found to be markedly higher in the inoculated RI than in the uninoculated RU seedlings (Fig. 2b). Localization of AOS abundance was very high in resistant seedlings when compared with the susceptible seedlings. The RI seedlings at 24 h post-pathogen inoculation showed maximum AOS abundance in vascular as well as in the epidermal regions (Fig. 2b). The treatment of the western as well as immuno-tissue blots with pre-immune serum and the secondary antibody alone resulted in no immuno-reaction.

Cloning of a fragment encoding putative pearl millet *PgAOS1* gene and comparative analysis of *PgAOS1* expression levels in resistant and susceptible pearl millet cultivars with and without pathogen inoculation

A putative 512-bp *AOS* gene fragment (*PgAOS1*) was isolated using degenerate primers with the final aim of using it as a probe to compare the expression levels of the AOS encoding gene(s) in the resistant (IP18292) and susceptible (7042S) pearl millet seedlings following pathogen inoculation. Alignment of the deduced amino acid sequence of *PgAOS1* with AOSs from other plant species showed the *PgAOS1* to encode the conserved CypX domain (Supplementary Fig. 1). Subsequently, an unrooted phylogenetic tree was constructed to determine the evolutionary position of the PgAOS1 in relation to other known plant-derived AOSs. The monocot and dicot AOSs formed two separate clusters and the pearl millet PgAOS1 was clearly grouped along with the AOSs derived from monocot plants (Fig. 3).

Northern analysis using the 512-bp fragment as a probe was then used to assess the expression levels of the *PgAOS1* gene in resistant and susceptible pearl millet cultivars with and without pathogen inoculation. A marked accumulation of *PgAOS1* transcript was observed only in the RI cultivar (Fig. 4a). The *PgAOS1* transcript abundance increased from 3 h and reached its maximum at 24 h in inoculated IP18292 seedlings. As for the susceptible seedlings, constitutive expression levels of *PgAOS1* in the uninoculated susceptible SU samples were lower than those detected in respective uninoculated resistant RU samples (Fig. 4a, b).

Discussion

Plants have evolved remarkable defensive strategies to protect themselves against a wide range of pests, pathogens and abiotic stresses [39–46]. One of the most prominent early intracellular signaling events involved in triggering specific cell responses include changes in genes, proteins and metabolites of the octadecanoid pathway which has a central role in plant growth, development and defense [47, 48].

The role of AOS has been well established in wound responses [16, 17]. However, less effort has gone into the understanding of the role of AOS in induction of disease resistance against phyto-pathogens. In this study, we have provided evidence for the involvement of AOS during host-pathogen interaction in pearl millet-downy mildew system. The study of AOS activity levels in various pearl millet cultivars with differential resistance to downy mildew disease showed a clear correlation between downy mildew disease incidence and AOS activity. The highly resistant and resistant pearl millet cultivars displayed significantly higher AOS activities than the susceptible and highly susceptible genotypes (Table 1; Fig. 1). Similar correlations have been established with other defense enzymes, such as lipoxygenase, polyphenol oxidase, phenylalanine ammonia lyase, glucanase, superoxide dismutase and peroxidase [22, 23, 25, 26, 28]. All these compounds together with AOS may serve as biochemical markers for screening of pearl millet varieties resistant for downy mildew disease. In addition, the induction of AOS activity followed by a time-course treatment of either resistant or susceptible genotype with *S. graminicola* clearly demonstrated the involvement of AOS in regulation of response to downy mildew disease in pearl millet.

In agreement with enhanced AOS activity, western blot analysis using polyclonal antibody raised against barley AOS also showed a strong support for the AOS activity

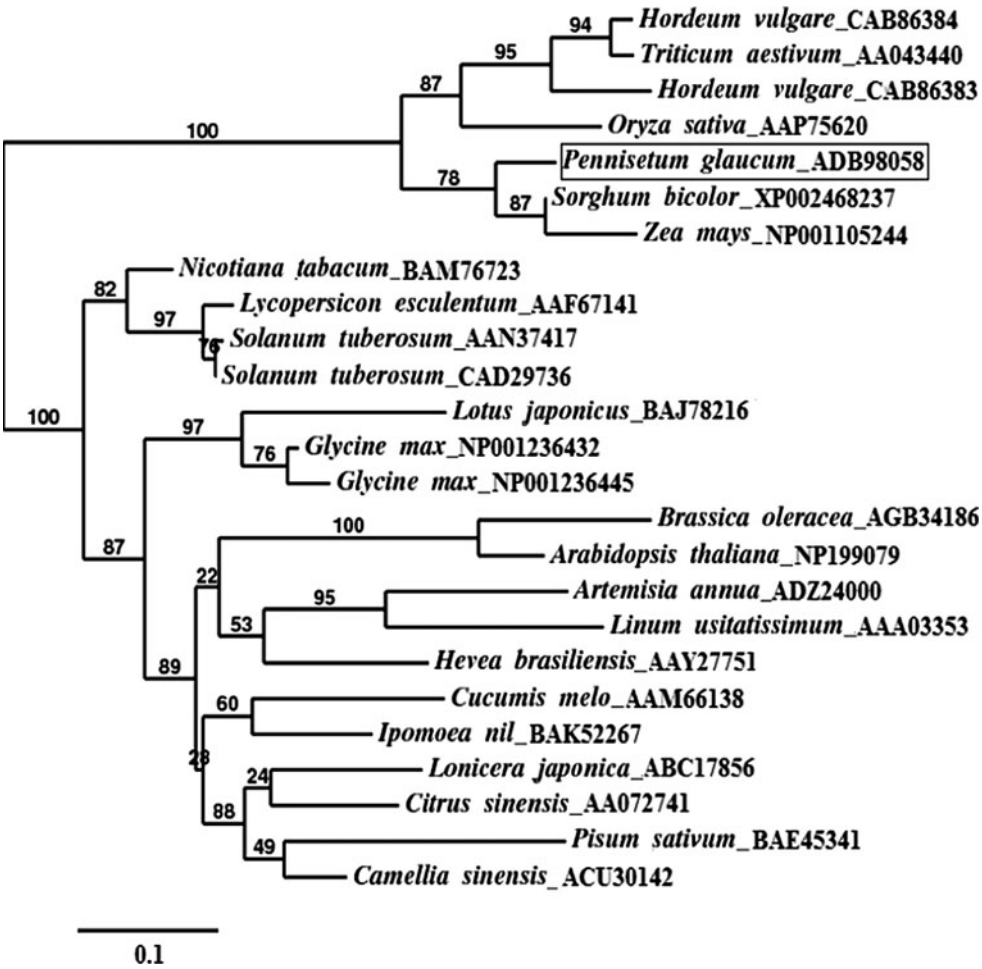

Fig. 3 Phylogenetic relationship of pearl millet PgAOS1 and other known AOSs (including CypX domains) from various monocot and dicot plants. Position of pearl millet AOS is highlighted by a *box*. The branch support values are represented at the branch points and the branch length scale is mentioned below the tree which indicates the relative divergence of the sequences examined. The protein IDs of AOS from various species are also displayed on the tree together with the species name

study as we detected marked induction of the examined AOS protein in the resistant pearl millet followed 24 h inoculation (Fig. 2a). The accumulation of AOS in pearl millet response to downy mildew disease was also investigated by the immuno-tissue blot technique, which is considered as a quick and easy method for detecting proteins in different plant tissues [49]. AOS abundance was higher in resistant inoculated than other tested seedling samples (Fig. 2b). Furthermore, the results obtained showed accumulation of AOS in both epidermal and vascular bundles. Thus, localization of enzyme in the epidermal and vascular tissues might be a part of the defense responses designed to prevent the entry as well as systemic spread of the pathogen through the vascular system.

The limited information of pearl millet genomic sequence has made gene discovery and molecular characterization much more difficult for this particular crop, when compared with other plant species that have their genomes completely sequenced. As an effort toward identification and functional analyses of the pearl millet AOS encoding genes, we have made an attempt to search for and clone *PgAOS* gene(s) from pearl millet. In the present study, we were able to clone a 512-bp fragment, which was identified as a part of the so-called *PgAOS1* gene. Sequence alignment and phylogenetic analysis demonstrated without any ambiguity that the *PgAOS1* is a plant AOS encoding gene (Fig. 3; Supplementary Fig. 1). More interestingly, expression analysis of *PgAOS1* revealed that its expression patterns were well correlated with the AOS levels and activities. Specifically, the *PgAOS1* was induced in pearl millet cultivars treated with *S. graminicola*, especially in incompatible cultivar in which higher expression levels of *PgAOS1* could be detected under both normal and treated conditions (Fig. 4). The AOS encoding genes have been shown to be induced in several plant species by pathogens. Expression of the *OsAOS2* in rice leaves was upregulated significantly upon *Magnaporthe grisea* infection [20]. A putative *AOS* gene was also reported to be induced in pea by a virulent pathogen *Mycosphaerella pinodes* 12 h after inoculation [10].

The finding of the close relationship between AOS levels and downy mildew resistance presented in this study indicates that AOS can potentially be used as a marker to detect the resistance or susceptibility of pearl millet

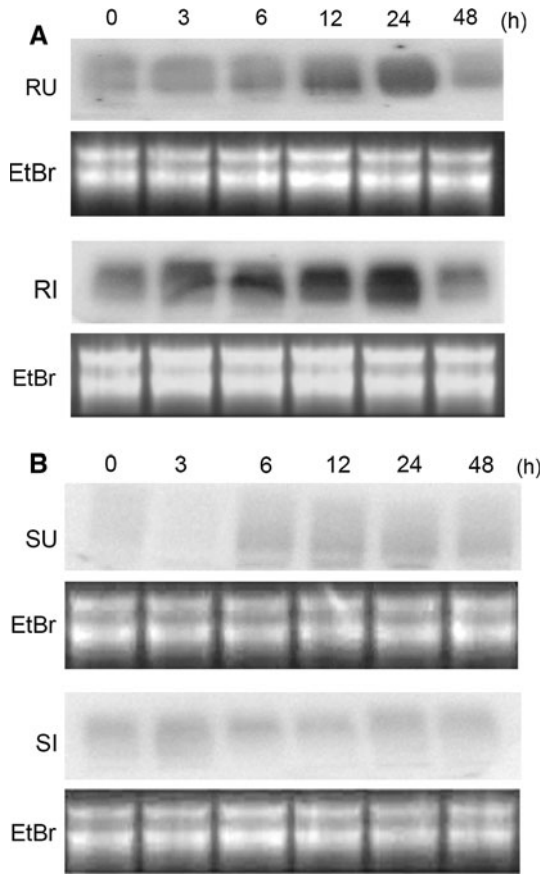

Fig. 4 Comparative expression analysis of *PgAOS1* gene in resistant and susceptible cultivars. **a** Northern blot analysis of *PgAOS1* in IP18292 resistant cultivar treated with water (RU, control) or *S. graminicola* (RI) for indicated time points. **b** Northern blot analysis of *PgAOS1* in 7042S susceptible cultivar treated with water (SU, control) or *S. graminicola* (SI) for indicated time points. Each lane was loaded with 20 μg of the total RNA. The blot was hybridized with the 512-bp α^{32}P-labled *PgAOS1*partial cDNA probe. *EtBr* ethidium bromide staining

varieties to downy mildew disease. Further functional analyses of AOS encoding gene(s) and other intermediary components in contrasting pearl millet cultivars during *S. graminicola* infection will be crucial for in-depth understanding of the involvement of the octadecanoid pathway in pearl millet-downy mildew interaction as well as provide candidate genes for development of pearl millet cultivars with enhanced resistance to downy mildew disease by genetic engineering. Sequencing of the whole genomic sequence of pearl millet would fasten the research on gene discovery and functional and comparative genomics of this important crop.

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Conflict of interest The authors declare no competing interests.

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