

BRIEF COMMUNICATION

Spatial and temporal dynamics of peroxidase and amine oxidase activity is linked to polyamines and lignin in wheat grains

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Abstract

The regulation of contents and activities of peroxidase (POX), diamine oxidase (DAO) and polyamine oxidase (PAO) were determined in relation to polyamines and lignin content in wheat (*Triticum aestivum* L.) grains. Two cultivars WH 542 (heat susceptible) and PBW 343 (heat tolerant) were used. Activities of POX, DAO and PAO were substantially higher in PBW 343 as compared with WH 542 and appeared to be independently regulated. POX and PAO showed peak activities at mid-milky stage (15 d post anthesis) while the activity of DAO showed continuous decline. Histochemical localization of POX and PAO *in situ* revealed their presence in the chalazal cell walls, crease and seed coat. Substantially higher activities of enzymes in PBW 343 correlated well with a higher degree of lignification in the chalazal cells as compared to WH 542.

Additional key words: diamine/polyamine oxidase, lignification, *Triticum aestivum*.

Entry of assimilates into the developing grain is initially *via* the phloem pathway and then *via* a post-phloem pathway (Patrick and Offler 2001). The phloem pathway extends from the rachis, through the base of the grain, and along the vascular bundle of the pericarp in the crease, also known as the groove or furrow. In the post-phloem pathway, assimilate passes through several other layers of parenchymatous cells, the chalazal cells, the nucellar projection, the endosperm cavity and finally the crease aleurone before entering the cells of the starchy endosperm. In wheat, the chalazal cells are thin walled during early grain developmental period, *i.e.*, between 12 and 18 days post anthesis (DPA) but become lignified during later stages of grain development (Cochrane *et al.* 2000). The deposition of lignin in the chalazal cell walls suggests that peroxidase (POX; EC 1.11.1.7) enzymes may play a significant role in cell differentiation which takes place in the crease region during grain development. POXs are monomeric heme-containing enzymes that are usually glycosylated catalyzing a large variety of reactions and are involved in lignification,

indole-3-acetic acid oxidation, cell wall polysaccharide cross-linking and oxidation of cinnamyl alcohols (Quiroga *et al.* 2000). POXs, however, require H₂O₂ for activity (Bogdanovic *et al.* 2008) and the source of H₂O₂ during seed ontogeny is not sufficiently known.

Diamine oxidase (DAO; EC 1.4.3.6) and polyamine oxidase (PAO; EC 1.4.3.4) play a major role in the catabolism of polyamines (PAs) in plant tissues. DAO catalyzes the oxidative deamination of the diamines putrescine (Put) and cadaverine, producing the corresponding amino-aldehyde, ammonia and H₂O₂ (Cona *et al.* 2006). PAO catalyzes oxidative deamination at the secondary amino group of PAs such as spermidine (Spd) or spermine (Spm) producing H₂O₂, 1,3-diaminopropane and 1-pyrroline (Martin-Tanguy 2001). Structurally, these two enzymes are different; DAO is a copper-protein containing pyrroloquinolinequinone as cofactor and PAO is a flavoprotein. DAO is widespread among the *Leguminosae* as well as in other taxonomic groups, while PAO has been apparently detected only in the *Graminae* and *Pontederaceae*. Histochemical

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Abbreviations: DAO - diamine oxidase; DPA - days post anthesis; PA - polyamine; PAO - polyamine oxidase; PCA - perchloric acid; POX - peroxidase; Put - putrescine; ROS - reactive oxygen species; Spd - spermidine; Spm - spermine; TLC - thin layer chromatography.

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localization studies have indicated that both DAO and PAO are cell wall localized enzymes (Slocum and Furey 1991) and since each produce H_2O_2 as a reaction product, their activities could exert a profound influence on plant growth through coupled H_2O_2 -dependent, POX-mediated cross-linking of soluble phenolics and isodityrosine moieties of extensions, lignin biosynthesis and auxin oxidation in cell wall. If so, an examination of the changes in the activity pattern of POX and amine oxidases, alongwith their histochemical localization in relation to lignin deposition might indicate a novel role in modulating assimilate supply in developing grains of heat tolerant and susceptible cultivars of wheat. In view of the heat stress induced thermotolerance, the developing grains of two wheat cultivars were monitored to determine if the levels of endogenous polyamines could be correlated with the observed response.

The enzymes used in microscopy and the substrates used to determine POX, DAO and PAO activities and PAs were all supplied by the *Sigma-Aldrich*, Poole, UK. Two cultivars of wheat (*Triticum aestivum* L.), PBW 343 (heat tolerant) and WH 542 (heat susceptible), were grown in the fields of Punjab Agricultural University, Ludhiana, Punjab, India following recommended agronomic practices. The mean maximum and minimum temperatures during vegetative stage and grain development were 34.8 and 15.6 °C, respectively. PBW 343 grows better in the climatic conditions of the Punjab as compared with WH 542. The susceptibility of WH 542 was measured from the heat susceptibility index which was calculated from grain yield per plot and thousand grain mass as described by Sumesh *et al.* (2008). In addition, physiological and grain yield parameters were also taken into account for characterizing cultivars for heat tolerance. Uniformly growing plants were tagged at anthesis and grain samples for enzyme assays and histochemical studies were collected at 7, 11, 15, 20, 30 and 40 d post anthesis (DPA). Grains (about 2 g, depending on the stage of development) were homogenized (triplicate samples) at 4 °C in 100 mM K-phosphate buffer (pH 6.5) containing 5 mM dithiothreitol and the extract centrifuged at 16 000 g for 20 min at 4 °C. The supernatant was used as source of enzyme. POX was assayed by the method of Claiborne and Fridovich (1979). DAO and PAO activities were estimated by a method based on the colorimetric assay of Δ -pyrroline using Put (for DAO) and Spd (for PAO) as substrates (Holmstead *et al.* 1961). For DAO and PAO activities, the reaction mixture of 2.0 cm³ consisted of 0.1 cm³ of enzymic extract, 50 units of catalase, 0.1 % *o*-aminobenzaldehyde and the reaction started with one of the two different buffer and substrate combinations, *i.e.* 10 mM Put in 50 mM K-phosphate buffer (pH 7.5) for DAO; 10 mM Spd in 50 mM K-phosphate buffer (pH 6.0) for PAO. The mixture was incubated at 30 °C for 3 h, and then stopped with 2.0 cm³ of 10 % (v/v) perchloric acid (PCA) and the tubes centrifuged at 6 500 g for 15 min. Formation of the Δ -pyrroline

product was determined by reading the absorbance at 430 nm. Control reactions were carried out with inactivated enzyme prepared by heating for 20 min in a boiling water bath. The conditions of the assays were optimized so as to give linear rates with respect to incubation time, optimum pH and substrate concentration.

Histochemical localization method infers the utilization and production of H_2O_2 as a result of POX, DAO or PAO activities through the development of a colored product from the oxidation of an artificial substrate of POX by H_2O_2 in the presence of endogenous or exogenous POX (Angelini and Federico 1989). Fresh, hand-cut sections cut transversely through the crease region of grains were washed with 0.05 M K-phosphate buffer, pH 6.5 (for POX) or 7.5 (for DAO) or 6.0 (for PAO). Sections were then incubated in the same buffer (1 cm³) containing 0.005 cm³ of 3-amino-9-ethyl carbazole in 0.1 M acetate buffer (pH 5.0). This artificial substrate is oxidized by POX in the presence of H_2O_2 yielding red or brown compounds. Following 5 min incubation at room temperature, 0.01 cm³ of Put or Spd solution (0.2 M in H_2O) was added, and sections incubated for 2 h at room temperature. Stain development was observed using an *Olympus AH3-RFCA* (*Olympus*, Tokyo, Japan) microscope. Control sections were treated in exactly the same way and at the same time except that Put or Spd were omitted. POX activity was visualized by replacing Spd or Put with H_2O_2 using 0.01 cm³ of a 2 mM H_2O_2 solution in water. Enzyme activity was found to be very sensitive to pH and substrate concentration. Thus the pH, incubation times and substrate concentrations used, were those which were found to give optimal color development. Additional controls were carried out in the absence of exogenous POX and in the absence of the artificial substrate 3-amino-9-ethyl carbazole. Enzyme localization was repeated several times. Photographs were taken on *Kodak Ectachrome* film. Detection of lignin was performed by phloroglucinol/HCl staining (Bate *et al.* 1994). Sections of wheat grains were immersed in 10 % phloroglucinol for 2 min followed by immersion in concentrated HCl for 1 min, and finally washed in 75 % glycerol for 5 min.

Polyamines were extracted in triplicate using a modification of the method described by Goren *et al.* (1982). Developing grains at 6, 12, 25 and 40 DPA were extracted in 5 % PCA on ice using 100 mg tissue per cm³ PCA. The homogenate was left on ice for 60 min and then was centrifuged at 10 000 g for 15 min. The supernatant contained free amines and the bound amines in soluble form while the pellet contained insoluble amines in the bound form. The bound amines in the supernatant were released by treating the fractions with 6 M HCl at 110 °C for 18 h in a sealed vial. After heating, the samples were filtered through glass wool, dried under a stream of air at 80 °C, and resuspended in PCA. The fractions were used for polyamine analysis and stored in plastic tubes at -20 °C. Samples were found to remain stable under these conditions for at least two months. PCA extracts were analyzed for free polyamines

following dansylation using a modification of an earlier method (Sieler and Wiechmann 1967). The dansylation mixture consisted of 0.2 cm³ PCA extract, 0.4 cm³ dansylchloride [*Sigma*, 5 mg cm⁻³(acetone)], and 0.2 cm³ saturated Na₂CO₃. After overnight incubation at room temperature and an addition of 0.1 cm³ proline (100 mg cm⁻³) to remove excess dansyl chloride, dansyl-polyamines were extracted in 0.5 cm³ of ethyl acetate by vortexing for 15 - 30 s. The organic layer containing the polyamines was separated by low speed centrifugation (3 000 g), removed and dried under nitrogen and 0.05 cm³ were used for spotting onto *LK 60 Whatman* silica gel thin layer chromatography (TLC) plates. Authentic polyamine standards (Put, Spd and Spm obtained from *Sigma* as hydrochlorides) were separated simultaneously by ascending chromatography. Identification was accomplished by comparison of Rf values obtained by TLC using the solvent system: cyclohexane:ethylacetate (5:4, v/v), followed by chloroform:triethylamine (25:2, v/v). Dansyl polyamines were quantified in triplicate on silica gel plates using a densitometer with excitation 360 nm and emission at 500 nm. Data was statistically analyzed using factorial randomized block design. The differences were considered significant at $P \leq 0.05$.

POX and PAO activities increased until mid-milky stage (15 DPA) of grain development and thereafter decreased until 30 DPA but increased again towards grain maturity, *i.e.*, at 40 DPA. However, DAO activity showed a continuous decline from initial high values until grain maturity and was much lower in comparison with PAO. Irrespective of stage of grain development, activities of these enzymes remained higher in PBW 343 as compared with WH 542 (Table 1). It appears that these enzymes

play specific roles during grain development. The considerably higher activities of these three enzymes in the PBW 343 make this genotype probably more tolerant than WH 542. An increase in POX and amine oxidase enzymes has been associated with tolerance to high temperature and water stress in crop plants (Sairam *et al.* 2000, Suzuki and Mittler 2006, Gong *et al.* 2008). Greater increase in POX activity at 15 DPA as reported here was also observed by Almeselmani *et al.* (2006) under late and very late planting conditions of wheat indicating induction of enzymes in response to increasing temperature.

Transverse sections incubated in K-phosphate buffer containing H₂O₂, and 3-amino-9-ethyl carbazole developed a reddish brown colour for POX activity in the funiculus-chalazal and nucellar projection regions, and appeared almost black in PBW 343 in contrast to the outer cell walls of aleurone and pericarp of WH 542. Apparent histochemical PAO activity showed an identical distribution. DAO activity appeared as red colour in the funiculus-chalazal cells in WH 542 and as a reddish brown colour in the PBW 343 (Fig 1). In contrast, no colour developed in the absence of exogenous substrate for these enzymes. Sections appeared coloured at other pHs also but the intensity of the colour was faint. Involvement of the vascular bundles, crease, chalazal and nucellar projection for the movement of nutrient substances into the endosperm of developing wheat grain was earlier reported by Asthir *et al.* (2002). Higher activities of POX and PAO and their co-localization in cell walls of the funiculus-chalazal, crease and seed coat regions of PBW 343 (Fig. 1) over the period from 7 - 15 DPA suggests their active involvement in the production and catabolism of H₂O₂ in this cultivar. The intensity of

Table 1. Activities of POX [$\Delta A_{430} \text{ g}^{-1}(\text{f.m.}) \text{ min}^{-1}$], DAO and PAO [$\text{pmol}(\Delta\text{-pyrroline}) \text{ g}^{-1}(\text{f.m.}) \text{ min}^{-1}$], and contents of Put, Spd and Spm [$\text{nmol g}^{-1}(\text{f.m.})$] of developing wheat grains at different DPA. Means \pm SE, $n = 6$ for POX, DAO and PAO; $n = 3$ for Put, Spd and Spm.

Parameter	Cultivar	7	11	15	20	30	40 DPA
POX	WH 542	7.5 \pm 0.3	12.2 \pm 0.2	13.2 \pm 0.3	9.6 \pm 0.1	3.2 \pm 0.1	6.8 \pm 0.1
	PBW 343	12.5 \pm 0.2	15.9 \pm 0.3	22.1 \pm 0.8	16.3 \pm 0.2	9.3 \pm 0.2	10.6 \pm 0.2
DAO	WH 542	184.3 \pm 6.2	151.2 \pm 4.1	140.2 \pm 3.2	84.8 \pm 2.8	35.6 \pm 1.3	32.6 \pm 2.6
	PBW 343	472.3 \pm 4.3	370.6 \pm 3.8	220.4 \pm 4.6	131.3 \pm 1.9	92.2 \pm 2.7	84.5 \pm 3.2
PAO	WH 542	82.3 \pm 1.4	183.3 \pm 6.9	252.8 \pm 6.6	121.6 \pm 5.2	74.7 \pm 3.6	90.4 \pm 2.7
	PBW 343	825.6 \pm 9.2	1018.6 \pm 10.9	1120.6 \pm 9.3	455.2 \pm 4.4	117.4 \pm 6.2	125.2 \pm 1.7
Parameter	Cultivar	6	12	25	40 DPA		
Put	WH 542	104.4 \pm 2.7	210.4 \pm 3.8	360.7 \pm 4.8	391.2 \pm 6.7		
	PBW 343	127.2 \pm 3.2	293.2 \pm 4.2	410.3 \pm 5.1	485.4 \pm 6.3		
Spd	WH 542	75.8 \pm 3.9	54.1 \pm 2.7	37.1 \pm 2.1	28.3 \pm 1.8		
	PBW 343	91.1 \pm 4.6	75.5 \pm 3.8	58.8 \pm 2.3	46.1 \pm 1.8		
Spm	WH 542	32.5 \pm 1.3	10.3 \pm 0.6	7.2 \pm 0.3	7.3 \pm 0.4		
	PBW 343	49.7 \pm 2.3	33.4 \pm 1.7	18.2 \pm 2.3	11.1 \pm 1.4		

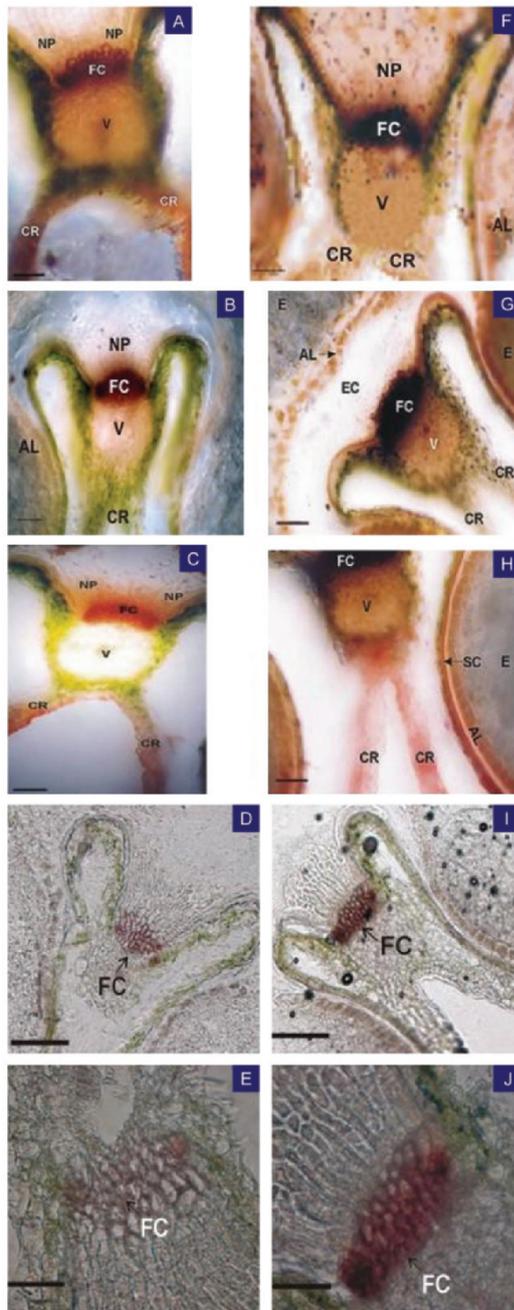


Fig 1. Histochemical detection of POX, DAO, PAO activities and lignin content in the developing grains of wheat WH 542 and PBW 343 at mid-milky stage (15 DPA). A, F - POX; B, G - DAO; C, H - PAO; D, E, I, J - lignin in the developing grains of WH 542 (A, B, C, D, E) and PBW 343 (F, G, H, I, J), respectively. AL - aleurone, CR - crease, E - endosperm, FC - funiculus-chalazal, NP - nucellar projection, V - vascular tissue, SC - seed coat. Bars = 900 μm (A, B, C, F, G, H), 650 μm (D, I), 2000 μm (E, J).

the colour for these enzymes was high in PBW 343 as compared to WH 542 and it coincided with maximum lignin content in the chalazal cells walls of PBW 343 (Fig. 1). Since amine oxidases produce H_2O_2 while POX

utilizes it for cross-linking reactions, it is therefore quite likely that these two enzymes are linked and may play an important role in deposition of cell wall materials like lignin that modulates assimilate entry into the grain. This correlation was earlier reported in barley (Cochrane 1983, 2000), light grown chick-pea stems (Angelini *et al.* 1990) and seedlings of *Pisum sativum* (Luhova *et al.* 2003). Both anionic and cationic POXs are involved in lignin deposition (McDougall 1992, Quiroga *et al.* 2000).

The deposition of lignin in chalazal cells of wheat at 15 - 18 DPA will restrict assimilate entry into the developing endosperm through symplast, while flow through the apoplastic pathway continues (Cochrane *et al.* 2000). Since in developing grains sucrose is supplied via the apoplast through cleavage by an extracellular invertase (Roitsch and Gonzalez 2004), lignifications is not expected to interfere with sugar supply. In addition, it is assumed that it is only the metabolic phase of assimilates within the grain that affects crop productivity as supply of assimilates is never the limiting factor under high temperature stress (Bhullar and Jenner 1986). Sumesh *et al.* (2008) associated high temperature tolerance of a cultivar with higher catalytic efficiency (V_{max}/K_m) of soluble starch synthase at elevated temperature and higher content of heat shock protein 100. A wall-localised increased production of H_2O_2 is in fact needed to sustain lignification as well as in cell wall stiffening events (De Marco and Roubelakis-Angelakis 1996, Cona *et al.* 2003) which probably act as a barrier during adverse environmental conditions as observed in cv. PBW 343. Higher lignification content in the outer aleurone layer of cv. PBW 343 helps in protecting the crop from adverse environmental conditions which in turn influences crop production. The strong up-regulation of PAO in chalazal cells and their direct correlation with POX (Table 1) could make PAO an attractive candidate for a role in the generation of H_2O_2 for wall-bound POX-mediated cross-linking and/or lignification in wheat.

The content of Put increased in grain of both cultivars from 6 to 40 DPA while Spd and Spm levels continuously declined till grain maturity (Table 1). The heat susceptible genotype reached a maximum Put content of 391 nmol g^{-1} (f.m.) while the tolerant cultivar attained a 19 % higher Put content of 485 nmol g^{-1} (f.m.) at 40 DPA. Put was the major polyamine present at all stages while the concentrations of Spd and Spm were considerably lower, their cumulative concentration reached only 9.1 % (WH 542) and 11.8 % (PBW 343) of the Put concentration at day 40. Accumulation of PA has been correlated to stress tolerance in plants and in improving embryogenesis (Hema and Murthy 2008). For instance, higher content of Put in the tolerant cultivar has probably led to an increase in POX or amine oxidase activities, producing H_2O_2 and aminoaldehydes as a physiological necessity during high temperature stress. Verma and Mishra (2005) reported that PAs could be multifaceted in nature, working as antioxidants, a free radical scavengers and a membrane stabilizers (Van den

Abeele *et al.* 2003). PAs can bind to negatively charged groups in the cell membrane so that phase change following stress treatment could be buffered.

The present study demonstrates that in the tolerant cv. PBW 343 a higher lignin deposition correlates with high histochemical POX and PAO activity. The active

role of POX in the seed coat may be to provide a protective shield to the grain through deposition of lignin that strengthens the cell wall which may not be that prominent in the susceptible cultivar. In contrast, any role of DAO in this process may be meagre as very little activity was detected in the crease or seed coat.

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