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POLYAMINE OXIDASE OF PRIMARY LEAVES IS APOPLASTIC IN OATS BUT SYMPLASTIC IN BARLEY

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Key Word Index—*Avena sativa*; oat; *Hordeum vulgare*; barley; Gramineae; cell wall enzymes; apoplast; protoplast; polyamines.

Abstract—Peeling away the lower epidermis and vacuum-infiltrating six-day-old primary leaves with buffered 200 mM NaCl extracted an intercellular washing solution (IWS) containing up to 99% of the polyamine oxidase (PAO) activity from oats, none of the PAO activity from barley, and no cytoplasmic glucose-6-phosphate dehydrogenase (G6P-D) from either species. In barley, but not oats, PAO activity was recovered from either isolated mesophyll protoplast or leaves from which the IWS had been extracted. We conclude that PAO is apoplastic and weakly ionically bound to mesophyll cell walls of oat primary leaves, but symplastic in primary leaves of barley. Implications of this differential localization including the involvement of PAO in providing hydrogen peroxide for lignification are considered.

INTRODUCTION

In the Gramineae the polyamines spermine and spermidine are oxidized by polyamine oxidase (PAO; EC 1.4.3.4) to produce diaminopropane, a pyrroline, and hydrogen peroxide. In legumes diamine oxidase (DAO; EC 1.4.3.6) typically oxidizes polyamines to a pyrroline, ammonia and hydrogen peroxide [1]. Polyamines are ubiquitous in the plant kingdom [1] and it has been suggested [2] that hydrogen peroxide produced by their oxidation might play a role in peroxidase-mediated reactions of lignification [3] if PAO and DAO were present in the apoplast; that is, outside the plasmalemma and associated with the cell wall. PAO has been reported to be apoplastic in oat primary leaves since activity is absent from mesophyll protoplast and about 80% of the total leaf activity was detected in the cell wall debris produced during protoplast isolation [4]. Most of the DAO activity in pea epicotyls could be extracted into an apoplastic intercellular washing fluid by centrifuging buffer-infiltrated segments [2]. In contrast, PAO activity in young barley leaves was associated with a particulate fraction which was presumably to be nuclei [5–7] although later experiments suggested that this might be an artifact of enzyme preparation [8]. In this study we report the distribution of PAO activity between apoplastic intercellular washing solutions, mesophyll protoplasts, and purified cell walls, in primary leaves of six-day-old oat and barley seedlings.

RESULTS AND DISCUSSION

Oat leaf polyamine oxidase

PAO with high specific activity was extracted from the apoplast of oat primary leaves, without contamination by the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6P-D), by peeling away the lower epidermis and vacuum-infiltrating with 5 mM K-Pi, pH 6.5, containing 200 mM NaCl. Infiltration for 60 min released no detectable G6P-D but extracted almost all of the PAO activity from the leaf (Table 1). This suggests that our technique is specific for apoplastic constituents and effective for extracting soluble and weakly ionically bound proteins from leaves from which the epidermis can be peeled with minimal damage to the underlying mesophyll. The intercellular washing solution (IWS) obtained during the first 12 min contained only *ca* 1% of the total soluble protein of the segment but the specific activity of PAO in this fraction was 1.25 μkat/μg protein (Table 1). In contrast, PAO purified from whole shoot homogenates of oats by precipitation and gel filtration had a specific activity of only 0.006 μkat/μg protein [6].

PAO in the apoplast of oat leaves is poorly soluble in buffer alone since a preliminary infiltration for 36 min with 5 mM K-Pi pH 6.5 extracted only about 2.5% of the total peeled leaf activity (Table 2). When the segments were subsequently extracted with buffer containing increasing salt concentrations, 50 mM NaCl extracted 28% of the whole leaf activity; 100 mM NaCl, an additional 43% of the activity; and 200 mM NaCl, 21% more of the activity. These four IWS fractions contained *ca* 95% of the total PAO activity of the leaf (Table 2). When segments were repeatedly infiltrated (4 × 36 min) with buffered 200 mM NaCl, this extracted *ca* 99% of the whole-segment PAO activity from the leaf without contamination by G6P-D (not shown).

After the IWS was extracted, samples were homogenized in 50 mM K-Pi pH 6.5, centrifuged, and the supernatant assayed for symplastic (cytosolic plus vacuolar) activity. The supernatant contained all of the G6P-D activity of the segment but only *ca* 4% of the PAO activity (Table 2). From our earlier experiments we consider that the low level of PAO activity recovered from the symplastic compartment was void-volume apoplastic enzyme not eluted from the leaf during a single 36 min infiltration wash. Only traces of PAO activity

Table 1. Effect of infiltration time on polyamine oxidase activity washed from the apoplast of six-day-old oat primary leaves

Apoplastic activity extracted Infiltration time (min)					Symplastic activity (retained in leaf after 60 min of extraction
12	24	36	48	60	
Polyamine oxidase (μkat H_2O_2)					
per segment					
6.9 ±0.4	9.2 ±0.6	10.3 ±0.2	11.5 ±0.5	13.9 ±0.9	0.91 ±0.18
per μg protein					
1.25	1.16	1.08	1.07	1.24	0.002
Glucose-6-phosphate dehydrogenase (nkat NADP/segment)					
nd*	nd	nd	nd	nd	0.50 ±0.02
Soluble protein (μg/segment)					
5.5 ±0.7	7.9 ±0.7	9.5 ±0.2	10.8 ±0.9	11.2 ±0.7	530.0 ±26.7

The lower epidermis was peeled from the leaf, 4 cm segments vacuum infiltrated with 5 mM K-Pi pH 6.5 containing 200 mM NaCl, and aliquots of the solution removed at times indicated. After 60 min, segments were removed, homogenized in 50 mM K-Pi pH 6.5, centrifuged, and the supernatant used as a source of symplastic (cytoplasmic plus vacuolar) enzymes. Values are means ± s.e.

*Not detected but the lower limit of detection was *ca* 0.005 μkat/segment.

Table 2. Sequential extraction of apoplastic and symplastic polyamine oxidase activity from six-day-old oat primary leaves

Fraction	Polyamine oxidase (μkat H_2O_2) per segment	per μg protein	% of total	Soluble protein (μg) per segment	% of total
IWS extracted by:					
buffer alone	0.41 ±0.05	0.08	2.5%	5.3 ±1.0	1.0%
+50 mM NaCl	4.53 ±0.04	1.42	28.0%	3.2 ±0.8	0.6%
+100 mM NaCl	7.00 ±0.10	2.41	43.3%	2.9 ±0.5	0.5
+200 mM NaCl	3.40 ±0.27	1.36	21.0%	2.5 ±0.5	0.5%
Ionically bound in cell wall	0.15 ±0.02	0.03	0.9%	4.8 ±0.2	0.5%
Covalentaly bound in cell wall	<0.01	*	<0.01%	*	
Symplastic supernatant	0.67 ±0.11	0.001	4.1%	517.3 ±5.7	96.5%

Segments were prepared as in Table 1. Intercellular washing solutions (IWS) were sequentially extracted by infiltration for 36 min with 5 mM K-Pi buffer, then for additional 36 min periods with buffer plus NaCl as shown. After the final 200 mM NaCl IWS was extracted, segments were removed, homogenized in 50 mM buffer, centrifuged, and the supernatant used as a source of symplastic activity. The pellet was washed with 1% (v/v) Triton X-100, then with buffer, and suspended in buffer containing 1 M NaCl to solubilize enzymes ionically-bound to the cell wall. Salt-extracted cell wall pellets were assayed for covalently bound activity. Values are means ± s.e.

*Protein could not be determined in these Cellulysin-digests.

Table 3. Comparison of polyamine oxidase activity in protoplasts, residual protoplast digesting medium, and freshly-peeled leaf segments, of six-day-old oat and barley primary leaves

		Supernatant of peeled leaf segment homogenized in:	
Protoplasts	Residual protoplast digesting medium	100 mM pH 6.5 K-Pi buffer	buffer plus 1 mM NaCl
Barley (nkat H_2O_2/segment)			
135 (98%)	nd*	111 (80%)	138 (100%)
Oat (μkat H_2O_2/segment)			
nd*	13.7 (84%)	7.9 (48%)	16.4 (100%)

Peeled leaf segments were incubated at 25° for 4 hr in 20 mM K-Pi pH 5.5 plus 0.6 M sorbitol and 1% (w/v) Cellulysin, and protoplasts separated from the digesting medium by filtration and centrifugation. Protoplasts were homogenized in 100 mM K-Pi pH 6.5, centrifuged, and soluble enzyme activity determined in the supernatants. Peeled segments were homogenized in 100 mM pH 6.5 K-Pi or in this buffer plus 1 M NaCl. Values (nkat for barely, μkat for oats) are means of three or more determinations.

*Not detected, but the lower limit of detection was *ca* 3.0 nkat/segment.

were detected in the 1 M NaCl extract or Cellulysin digest of purified cell walls (Table 2).

To indirectly confirm the apoplastic localization of oat leaf PAO, we prepared mesophyll protoplasts and determined activity in the protoplasts and in the residual protoplast-digesting solution (including protoplast washes). No PAO activity was detected in oat protoplasts (Table 3) confirming previous reports [4]. The combined protoplast digesting solution and protoplast washes had 84% as much PAO activity as did the supernatant of segments homogenized in buffered 1 M NaCl (Table 3). Although sorghum seedlings contain a dialysable PAO inhibitor [9], Kaur-Sawhney *et al.* [4] reported that there are no PAO inhibitors in oat protoplasts. We found that dialysis did not increase PAO activity in any enzyme fraction from oats, and PAO activity in oat IWS was not inhibited by the addition of the crude supernatant from lysed protoplasts or whole-segment homogenates of oats.

Kaur-Sawhney *et al.* [4] reported that *ca* 80% of the PAO activity in oat leaves was associated with cell wall debris left in the digestion solution of oat mesophyll protoplast isolated in cellulase-containing osmoticum buffered with 1 mM K-Pi. We find that apoplastic oat leaf PAO is relatively insoluble in 5 mM K-Pi buffer alone (Table 2) and suggest that the activity which they found in the cell wall debris was weakly ionically bound to that fraction. Our direct extraction of essentially all of the PAO activity from the apoplast without contamination by cytoplasmic G6P-D (Table 1 and 2) and our confirmation of its absence from mesophyll protoplasts (Table 3) indicate that oat leaf PAO is exclusively apoplastic and weakly ionically bound to cell walls.

Barley leaf polyamine oxidase

There are striking differences in the localization of PAO in six-day-old primary leaves of oats and barley. PAO activity could not be detected in the IWS from barley (Table 3). However protoplasts isolated from barley leaves in a 0.6 M sorbitol osmoticum contained *ca* 98% of the activity in the supernatant of peeled leaves homogenized in 1 M NaCl (Table 3). About 4% of the barley PAO activity was recovered by extracting Triton-X 100 washed cell-walls with 1 M NaCl (Table 4).

As we had demonstrated that essentially all of the PAO in oat leaves could be extracted into the IWS, a major effort was made to detect PAO activity in the IWS from barley. We were unable to detect PAO activity in the barley IWS by our standard spectrophotometric assay or by an oxygen electrode assay [10], although either assay was satisfactory for assaying PAO in the IWS from oats or in dialysed symplastic preparations from barley. We were also unsuccessful in our attempts to detect PAO in the IWS of barley after dialysis against distilled water or various buffers, after Sephadex G-25 filtration, by using different polyamine substrates, or by assaying for PAO activity at different pH values.

Smith [11] reported that the optimal pH for assaying barley PAO was 4.8, but commented that some protein precipitated in the reaction mixture under these conditions. When we attempted to assay barley PAO at pH 4.8 using enzyme preparations from either lysed protoplasts or whole-leaf homogenate, the reaction mixtures became turbid as the reaction proceeded and apparent PAO activity was highly variable between experiments. Neither dialysis nor gel filtration of the extracts corrected this problem. To resolve this we assayed barley PAO at a pH 5.5 which, although it may have underestimated the absolute activity of barley PAO [5], gave reproducible results and allowed us to compare PAO activity in different fractions.

Unless the barley symplastic enzyme preparations were dialysed, PAO activity was absent or highly variable, and at best *ca* 10% of that determined after dialysis. Undialysed barley homogenate was strongly inhibitory to PAO activity in dialysed barley preparations or to enzyme preparations from oats. Dialysed barley preparations were not inhibitory to oat PAO activity.

Although the PAO inhibiting fraction(s) from barley, could be removed by dialysis, enzymes solubilized by salt

Table 4. Polyamine oxidase activity extractable from apoplastic and symplastic compartments of six-day-old oat and barley primary leaves.

Activity in apoplastic/symplastic fractions			Whole-segment activity
Apoplastic: extracted in 200 mM NaCl IWS	Apoplastic: 1 M NaCl soluble from wall	Symplastic: in supernatant after IWS had been extracted	Supernatant of peeled segments homogenized in 1 M NaCl*
Barley (nkat H_2O_2/segment			
nd†	6.1 (4%)	119.9 (87%)	137.6 (100%)
Oat (μkat H_2O_2/segment)			
13.89 (85%)	0.57 (3%)	0.55 (3%)	16.43 (100%)

Leaves were peeled and vacuum-infiltrated for 36 min with 5 mM K-Pi pH 6.5 containing 200 mM NaCl to extract an IWS containing soluble and weakly ionically bound apoplastic activity, then homogenized in 50 mM K-Pi pH 6.5 and centrifuged to obtain a supernatant containing soluble symplastic activity. The pellet was washed with 1% Triton X-100, then with buffer, and the purified wall pellet extracted with 1 M NaCl to solubilize strongly ionically-bound apoplastic activity. Whole-segment activity was determined by homogenizing peeled leaves in buffer plus 1 M NaCl, centrifuging, and the supernatant used as a source of whole-segment enzyme activity. Values (nkat for barley, μkat for oats) are means of three or more replicates.

*Values from Table 3.

†Not detected, less than 3.0 nkat/segment.

solutions sometimes bind to membranes during dialysis [6, 12]. This may be a general problem when working with weakly ionically bound enzymes from the apoplast. For example, apoplastic oat PAO is poorly soluble in 5 mM K-Pi buffer alone (Table 1) and when the 200 mM NaCl IWS from oats was dialysed against distilled water, about 40% of its PAO activity was lost, presumably by binding onto the dialysis membrane (not shown).

Comparison of PAO distribution between the apoplast and the symplast of oat and barley primary leaves. We determined the relative distribution of PAO activity in the apoplast and symplast of barley and oat leaves by the sequential processes of: (i) extracting soluble and weakly ionically bound apoplastic activity into the 200 mM NaCl-containing IWS, followed by (ii) removing the segments from the IWS and homogenizing them in 50 mM K-Pi pH 6.5 and centrifuging to obtain a supernatant containing symplastic activity and finally (iii) releasing strongly ionically bound PAO activity from washed cell wall pellets by extracting the walls with buffered 1 M NaCl. The PAO activity recovered in these three fractions was more than 90% of that recovered in the supernatant of freshly peeled segments homogenized in 1 M NaCl (Table 4). The missing PAO activity from these three fractions was presumably in the void volume of the cell wall pellet, plus a fraction bound to disrupted cytoplasmic constituents in the pellet, which would be lost into the Triton X-100 and buffer washes used to purify the final wall pellet. All of these results lead us to conclude that polyamine oxidase in primary leaves is apoplastic in oats but symplastic in barley.

CONCLUSIONS

It has been suggested that if PAO is a cell wall-associated enzyme, one could presume that its substrates would be located there [4]. Goldberg and Perdrizet [13] found that most of the polyamines of mung-bean hypocotyls were localized in the cytoplasm of young cells, but bound to pectic materials in the cell walls in older tissues. Preliminary results from this laboratory indicate that the IWS of oats contains polyamines and their oxidation products (Liu and McClure, in preparation). Federico and Angelini [2] suggested that hydrogen peroxide produced in the cell wall when polyamines are oxidized by PAO or DAO might be used in the peroxidative activation of cinnamoyl alcohols for lignification. The source of apoplastic hydrogen peroxide is controversial. Gross *et al.* [14] proposed that hydrogen peroxide could be produced in the cell wall by a NADH-driven reversal of the general peroxidase reaction using cell wall-bound malate dehydrogenase coupled with a malate-oxaloacetate shuttle across the plasmalemma. Malate dehydrogenase has been found in cell walls [14] but there is no conclusive evidence for NAD(H) in cell walls [15].

Although the presence of apoplastic PAO in oats [4, this paper] and apoplastic DAO in peas [2] supports the suggestion that these enzymes might produce hydrogen peroxide for lignification, PAO appears to be totally symplastic in six-day-old barley primary leaves (Tables 3 and 4). This difference suggests that the localization of PAO, DAO and their substrates should be examined in a range of lignifying tissues and that alternate mechanisms for the formation of hydrogen peroxide in the cell wall [14, 16] should be considered.

EXPERIMENTAL

Plant material. Barley (*Hordeum vulgare* cv Atlas 68) and oat (*Avena sativa* cv Victory) seedlings were grown on vermiculite at 23° under a 16 hr light (165 μE/m^2/sec from cool-white fluorescent lamps) 8 hr dark photoperiod and irrigated daily with 1/3 strength Hoagland's nutrient soln. Primary leaves were harvested on the sixth day after planting, 10 hr after the onset of light.

Extraction of intercellular washing solution. A transverse cut was made with a single-edged razor blade through the upper epidermis and mesophyll, *ca* 1 cm below the tip of the primary leaf, and the lower epidermis peeled away. The peeled leaves were

immersed in cold H_2O and gently agitated by hand for *ca* 45 sec to remove cytoplasmic contaminants of damaged cells. Apical 4 cm segments were cut and 5 (oats) or 10 (barley) segments placed in a cold 15 × 95 mm test tube. 5 ml of cold de-gassed 5 mM K-Pi, pH 6.5, containing NaCl as indicated (normally 200 mM) was added to the tube and the segments were vacuum-infiltrated at 91 kPa on ice for 36 min (unless otherwise indicated), breaking and re-establishing the vacuum every three to five min to facilitate infiltration and washing. The IWS extracted by this technique is free from symplastic contamination [17]. The IWS was used as a source of soluble and weakly ionically bound apoplastic enzymes.

Soluble cytoplasmic enzymes. Unless otherwise indicated, all subsequent fractionations were done at 4°. After the IWS was extracted, segments were homogenized in 5 ml 50 mM K-Pi pH 6.5, centrifuged at 27 000 *g* for 15 min at 4°, and the supernatant used as a source of soluble cytoplasmic enzymes.

Ionically-bound cell wall enzymes. The cell wall pellet was washed twice by centrifuging in 50 mM K-Pi pH 6.5 buffer plus 1% (v/v) Triton-X 100 and ×3 with buffer alone to remove traces of contaminating cytoplasmic enzymes. The washed pellet was resuspended in 2 ml of buffered 1 M NaCl, stirred on ice for 30 min, centrifuged at 27 000 *g* for 15 min, and the supernatant used as a source of enzymes strongly ionically bound to the cell wall.

Covalently-bound cell wall enzymes. The salt-extracted oat wall pellets were digested with Cellulysin (see below) to liberate covalently-bound cell wall enzymes. The barley pellet was assayed directly for covalently-bound enzyme activity since Cellulysin interfered with barley (but not oat) PAO assays.

Total PAO in peeled leaf segments. Peeled segments were homogenized in buffer plus 1 M NaCl, centrifuged as described above, and the supernatant used as a source of soluble plus ionically bound enzymes. Enzyme preparations from barley were dialysed for 6 hr at 4° against 50 mM MES pH 5.5. Dialysis against more acidic soln or H_2O pptd much of the barley proteins. Oat preparations were not dialysed (see results).

Protoplast isolation. Peeled leaf segments were incubated in 20 mM K-Pi pH 5.5 containing 0.6 M sorbitol and 1% (w/v) Cellulysin (Calbiochem) for 4 hr at 25°. The digest was filtered through a stainless steel screen to remove undigested veins and cuticles. Protoplasts were pelleted at 150 *g* for 4 min and washed 3 × by centrifuging with 100 mM K-Pi, pH 6.5, containing 0.6 M sorbitol. Recovery of protoplasts was *ca* 75% (oat) and 82% (barley) based on chlorophyll retention. Washed protoplasts were homogenized in 100 mM K-Pi pH 6.5 and centrifuged at 16 000 *g* 20–30 sec. Barley protoplast supernatant was dialysed as above. Oat protoplast supernatant was assayed without dialysis.

Enzyme assays. Polyamine oxidase (PAO) activity was determined by a procedure modified from refs [4, 11]. The 1 ml reaction mixtures contained 0.78 ml of either 200 mM NaOAc pH 5.5 (barley, see results) or 100 mM K-Pi pH 6.5 (oat), 0.05 ml of 25 mM guaiacol, 0.05 ml horseradish peroxidase (10 units, Sigma type II), 0.1 ml of enzyme and 0.02 ml of 25 mM spermine (barley [11]), or spermidine (oats [4]). Polyamines were omitted from the reference. Reaction mixtures were pre-incubated at 30° for 2 min, reactions initiated by adding polyamine, and enzyme activity detected by the increase in *A*470 nm. PAO activity was calculated by using an extinction coefficient of 4370/M H_2O_2/cm [18]. For confirmation of these results PAO activity was occasionally determined by the oxygen electrode assay described in ref. [10]. G6P-D was assayed by the method of ref. [19], protein by ref. [20].

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