A transient increase in apoplastic peroxidase activity precedes decrease in elongation rate of B73 maize (Zea mays) leaf blades

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Peroxidase (EC 1.11.1.7) activity from homogenized tissue or in apoplastic fluid was analyzed along the developmental gradient of expanding B73 maize (Zea mays L.) leaf blades. Soluble plus ionically bound peroxidase activity from homogenized tissue was present in high levels at the leaf base, which includes the region of cell division, and decreased as tissue was displaced away from the base by growth. A different pattern of change in peroxidase activity was seen in apoplastic fluid extracted from segments of intact tissue, where an increase in peroxidase activity preceded a rapid decrease in leaf elongation rate. Similar patterns in peroxidase activity from homogenized and intact tissue have been found in leaf blades of tall fescue (Festuca arundinacea Schreb.), suggesting a common phenomenon. At the location within the elongation zone where the increase in apoplastic peroxidase activity occurred, the activities of neutral and acidic (pI 4.6) peroxidase isoforms were also elevated in both the homogenate and in apoplastic fluid. The coincidence of these isoforms with the decline in leaf elongation rate suggests they may contribute to cessation of growth. At the distal end of the elongation zone, the activities of other acidic peroxidases (pI 5.6 and 5.7) increased in the homogenate and in apoplastic fluid, and remained elevated as tissue was displaced into the maturation region. The location of their appearance and their relatively high activity in the maturation region suggest the involvement of these isoforms in lignification.

Abbreviations – G6PDH, glucose-6-phosphate dehydrogenase; LER, leaf elongation rate.

Introduction

The functions through which peroxidase might influence plant growth include lignin synthesis (Siegel 1953), oxidative coupling reactions involving phenolics that are esterified to cell wall polysaccharides (Geissman and Neukom 1971, Fry 1982a), formation of isodityrosine bridges that are believed to crosslink structural protein molecules (Fry 1982b), and catabolism of IAA (Jacob and Bopp 1990).

An inverse relationship between growth rate and peroxidase activity has been demonstrated in many plant developmental systems. In etiolated pine hypocotyls, where growth rate decreased with displacement of tissue through the 20 mm proximal to the cotyledonary node (Lorences et al. 1990), apoplastic peroxidase activity increased with displacement of tissue away from the cotyledons, and ionically bound peroxidase appeared first to decrease, then also increased with displacement (Sánchez et al. 1995). Peroxidase from homogenized cotton (Gossypium barbadense) fibers increased as elongation rate slowed and stopped (Rama Rao et al. 1982, Thaker et al. 1986). In extracts from homogenized etiolated pea (Pisum sativum) hypocotyls, cessation of elongation was accompanied by an increase in the activity of anionic peroxidase isoenzymes (Gardiner and Cleland 1974). In etiolated maize coleoptiles, exposure to red light resulted in an increase in growth and a decrease in peroxidase activity, consistent with peroxidase-regulated growth (Kim et al. 1989); the far-red reversibility of this growth response indicated phytochrome regula-
tion, possibly by affecting the rate of secretion of peroxidase into the cell wall. Apoplastic peroxidase activity of red light-grown cucumber (*Cucumis sativus*) hypocotyls increased upon exposure to blue light, but in this case, peroxidase did not appear quickly enough to govern the initial decrease in elongation rate (Shinkle et al. 1992). However, other studies using cucumber hypocotyls have demonstrated that blue light reduces growth rate, but does not alter the mechanical properties of the wall that are thought to be affected by peroxidase (Cosgrove 1988).

Elongating grass leaf blades constitute a developmental sequence from meristematic tissue at the base to mature tissue at the tip, with readily defined regions of elongation and secondary cell wall deposition in between. This experimental system allows changes in enzyme activity and other factors to be accurately related to specific developmental stages. In leaf blades of tall fescue (*Festuca arundinacea* Schreb.), a C₄ grass, a decrease in leaf elongation rate occurred following a transient increase in apoplastic peroxidase activity (MacAdam et al. 1992b). The objective of the present study was to investigate the relationship between peroxidase activity in the cell wall and leaf elongation rate using a C₄ grass, maize inbred line B73.

**Materials and methods**

**Plant growth conditions**

Seed of maize (*Zea mays* L.) inbred line B73 was surface-sterilized (with 1.1% (w/v) available chlorine, then rinsed several times with tap water before being sown in 12-cm-deep by 15-cm-diameter pots filled with Redi-Earth Peat-Lite potting mix (W. R. Grace & Co., Cambridge, MA, USA). Constant light with an irradiance of 400 μmol m⁻² s⁻¹ at seedling level was provided by Sylvania 45 W cool white high-output lamps, and the temperature was maintained at 25°C. Relative humidity was 40%, and plants were irrigated daily to the drip-point using tap water.

**Growth measurements**

All experimentation was done using the emerged, elongating second true leaf blade at lengths from base to tip of 60 to 90 mm. At this stage, the ligule was differentiated but was not displaced from the leaf base, and elongation zone length was constant. For determination of leaf elongation rate (LER), increase in length of the second true leaf of 20 seedlings was measured daily for 3 days beginning when the tip of the leaf emerged from the enclosing leaf sheath. Measurements were taken from the base of the sheath of the first leaf to the tip of the second leaf.

Eleven expanding leaves were used for the determination of elongation zone length. The leaf was excised from the plant at the point of attachment of the sheath of the enclosing first leaf, and the second expanding leaf was gently pulled from inside the first leaf. A 4% (w/v) solution of polyvinylformaldehyde in chloroform (Schnyder et al. 1990) was spread with a small-tipped paintbrush over the abaxial surface of the leaf. When the chloroform had evaporated, a strip of clear celophane tape was applied to the leaf and the replica was lifted off and applied to a glass microscope slide. The length of 7 intercostal epidermal cells was measured at 2-mm intervals from the ligule to 40 mm along each leaf using a Zeiss axioskop microscope equipped with an eyepiece micrometer.

Displacement velocities within the elongation zone were calculated using the equation

\[ V_x = \frac{V_{\text{MAX}} L_x}{L_{\text{MAX}}} \]

where \( V_x \) represented displacement velocity (mm h⁻¹) at a specific location (X) distal to the ligule, \( L_x \) was the length of an epidermal cell (mm) at the same location, \( V_{\text{MAX}} \) was LER, and \( L_{\text{MAX}} \) was final epidermal cell length (Carmona and Cuadrado 1986, Schnyder et al. 1990).

Relative segmental elongation rate (mm mm⁻¹ h⁻¹) was obtained by differentiating a cubic equation fitted to displacement velocity as a function of distance above the ligule (Hejnowicz and Brodzki 1960, Schnyder et al. 1990). To express elongation rate as a function of duration of displacement from the ligule, displacement velocity at each location was divided by 2 mm, the fixed length of the interval that was used to take measurements along the leaf, and time intervals antecedent to a specific location were summed.

**Mitotic index**

Expanding leaf blades were sectioned at 2-mm intervals from the ligule to 20 mm, and segments were fixed in a 3:1 mixture of ethanol and glacial acetic acid for 1 h, then transferred to 70% ethanol. A single segment placed on a slide was teased apart in 45% acetic acid and smeared using a coverslip as described by Jensen (1962). The mitotic index was calculated as the percentage of cells with mitotic nuclei at stages of division between prophase and telophase (Ellis et al. 1983). This experiment had 7 replications.

**Total soluble plus ionically bound peroxidase activity**

For homogenization, the basal 50 mm of 15 expanding leaf blades was sectioned transversely at intervals of 5 mm. To assay total soluble plus ionically bound peroxidase (EC 1.11.1.7), leaf tissue was frozen in liquid nitrogen, ground in a mortar, and extracted with cold 50 mM phosphate buffer, pH 6.0 (Lee 1973), containing 1.5% (w/v) polyvinylpolypyrrolidone (Gegenheimer 1990), 1 mM benzamidine HCl, and 0.8 M KCl. The
homogenate was centrifuged at 16000 g for 30 min, and the supernatant filtered through Micropure-0.45 particle separators (Amicon, Beverly, MA, USA).

Peroxidase activity was assayed at 30°C in a reaction mixture containing 1 ml 0.1 M potassium phosphate buffer (pH 6.0), 0.0167 ml 0.2 M guaiacol, and 0.0133 ml 0.03 M hydrogen peroxide. The reaction was initiated by adding 0.01 ml extract, and increase in $A_{270}$ min$^{-1}$ was measured. Guaiacol was used as the substrate in this assay because it reacts with more isoforms of peroxidase than many other hydrogen donors (Ievinsh 1992), and because it remains in solution more readily than other substrates during the assay. Soluble protein in the homogenate was analyzed by the Bradford (1976) method. Total soluble plus ionically bound peroxidase activity and soluble protein content are reported on a tissue fresh weight basis. This experiment had three replications.

Cell wall peroxidase activity

Apoplastic extraction preserves cell structure and avoids the redistribution of peroxidase from the vacuole or cytoplasm to the cell wall that occurs upon homogenization (Mäder et al. 1986). The basal 64 mm of 30 expanding leaf blades was sectioned at intervals of 8 mm. Baskets from microfilterfuge tubes (Rainin Instrument Co, Woburn, MA, USA), with the filter membrane removed, were filled with segments from a given location. At the seedling stage, the basal leaf blade segments are very fragile, so to minimize handling, segments were rolled in a small plastic strip before being inserted into infiltration baskets. Leaf blade segments were rinsed three times with cold, distilled, deionized water for 5 min to remove contaminants from the cut ends of the sections. Leaf sections were submerged in cold 10 mM phosphate buffer, pH 6.0, containing 0.2 M KCl, and vacuum-infiltrated at a pressure of 30 kPa for 10 min.

After infiltration, baskets were blotted with lint-free tissues to remove excess buffer, transferred to microcentrifuge tubes, and centrifuged at 1000 g for 15 min at 4°C (Terry and Bonner 1980). Infiltration and centrifugation were repeated three times. Apoplastic fluid from successive infiltrations was pooled, then desalted and concentrated to 60 μl using Ultrafree-MC Microconcentrators (Amicon). After apoplastic fluid extraction, cell contents were extracted from leaf segments by boiling segments successively in ethanol and water (MacAdam et al. 1992b) for determination of insoluble (cell wall) dry weight.

Activity of glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), a cytoplasm marker, was assayed in apoplastic fluid at 30°C in a 1-ml reaction volume at a wavelength of 340 nm (Li et al. 1989). Ten μl of apoplastic fluid was added to a reaction mixture containing 68 mM Tricine (adjusted to pH 8.0 with NaOH), 6 mM glucose-6-phosphate, 0.1 mM MgCl$_2$ and 0.6 mM NADP. The functionality of this assay was first tested with purified enzyme and also with homogenate. All reagents used in this assay were from Sigma. No detectable G6PDH activity was found in apoplastic fluid extracts using this procedure.

The peroxidase assay was carried out as reported for the homogenate. Soluble protein in the apoplastic fluid was too dilute for the Bradford assay, and was therefore analyzed using the bicinchoninic acid protein assay (Smith et al. 1985) following a microtiter plate protocol (Pierce, Rockford, IL, USA). Apoplastic soluble plus ionically bound peroxidase activity and protein content are reported on an insoluble dry weight basis. This experiment had 5 replications.

Isoelectric focusing gels

Isoelectric focusing gels were prepared with 1% (w/v) Isogel agarose (FMC BioProducts, Rockland, ME, USA) and 12% sorbitol as recommended by FMC, and with ampholytes pH 3 to 10 (Pharmacia LKB, Piscataway, NJ, USA). Gels were cast by hand onto Gelbond film (FMC). The focusing gel for the homogenate was loaded with 6 μl sample per lane. On the gel used for detection of apoplastic peroxidase isoforms, because enzyme activity in apoplastic fluid was low, a total of 10 μl sample was loaded onto two masks positioned parallel to each other across the center of the gel. For both the homogenate and the apoplastic fluid, total extract from segments at each location along the leaf was concentrated to a constant volume, and an aliquot was loaded on the gel to allow comparison of activity per segment of leaf blade tissue.

Samples of the homogenate were prefocused for 10 min and focused for 1.5 h, and apoplastic fluid samples were prefocused for 1 h and focused for 1.5 h. Prefocusing was done at 1 W constant power and 1500 V, and focusing was done at 8 W constant power and 1500 V. To determine the pI of the peroxidase isoforms, pi markers in the range of 3.6 to 10.2 (FMC) were also run on each gel.

Both gels were run at 4°C and stained for peroxidase activity using p-phenylenediamine-pyrocatechol (Imberty et al. 1984). This activity stain is relatively less selective toward peroxidase isoforms than other hydrogen donors, and it precipitates rapidly and does not fade as the gel is dried.

Results and discussion

Mitotic index was highest at the base of the leaf blade, and decreased as tissue was displaced away from the base, reaching zero at about 10 mm distal to the ligule (Fig. 1). In wheat, cell division was also restricted to the basal 10 mm of the leaf blade (Ellis et al. 1983). In
studies of tall fescue, while mesophyll cell division occurred within the basal 10 to 15 mm of the elongating leaf blade depending on the genotype, epidermal cell division was limited to the basal 2 to 3 mm (MacAdam et al. 1989). In the fully expanded leaf, epidermal cells form a continuous tissue of elongated cells, while mesophyll tissue consists of smaller cells interspersed with air spaces. Average leaf elongation rate was 1.29 mm h\(^{-1}\) ± 0.12 se, \(n = 20\). Relative segmental elongation rate, which can be used to define the elongation zone of the leaf blade, increased from the ligule to a peak at around 10 mm distal to the ligule, and elongation stopped at approximately 22 mm (Fig. 1).

Total soluble plus ionically bound (extractable) peroxidase activity and soluble protein content in the homogenate were high at the leaf base and decreased to a minimum between 10 and 15 mm distal to the ligule (Fig. 2), similar to the change in soluble peroxidase activity from homogenized mung bean hypocotyls (Goldberg et al. 1986). Total extractable peroxidase activity and soluble protein in the homogenate remained low through the maturation region. This result suggested that total extractable peroxidase activity was associated in some way with cell division (Fig. 1). Specific activity of total extractable peroxidase was highest between 10 and 15 mm distal to the ligule (Fig. 2, inset); elongation rate peaked in the same region (Fig. 1).

In elongating tall fescue leaf blades, peroxidase activity from homogenized tissue followed a similar pattern (MacAdam et al. 1992a). Highest peroxidase activity and soluble protein were also noted in the homogenized youngest internodes of dwarf pea (Birecka and Galston 1970), and both decreased with elongation.

**Fig. 1.** Mitotic index (■) was obtained from tissue squashes of 2-mm-long leaf blade segments. Vertical bars represent ± se; data are the mean of 7 replications. Relative segmental elongation rate (□) was calculated as the first derivative of displacement velocity, which was determined from leaf elongation rate and the spatial distribution of epidermal cell lengths along the expanding second maize leaf blade.

**Fig. 2.** Spatial distribution of soluble plus ionically bound peroxidase activity (■) and soluble protein (□) in leaf blade homogenate. Vertical bars represent ± se; data are the mean of 3 replications, each containing 15 leaves. Inset: Specific activity of peroxidase in homogenate.

Total extractable peroxidase activity and soluble protein in the homogenate were expressed on a fresh weight basis, to better illustrate change in enzyme activity with differentiation of the leaf blade. Fresh weight per mm increased with growth and the associated uptake of water as tissue was displaced through the elongation zone, then decreased as the leaf blade narrowed toward the tip (Fig. 3).

Peroxidase activity in apoplastic fluid represents in vivo cell wall enzyme activity, and the pattern of change in soluble plus ionically bound peroxidase activity in the apoplast was very different from activity in the homogenate. As tissue was displaced through the elongation zone, apoplastic peroxidase activity increased to a peak between 8 and 16 mm distal to the ligule (Fig. 4), approximately the same location at

**Fig. 3.** Fresh weight (■) and insoluble dry weight (□). Vertical bars represent ± se; data are the mean of 3 replications, each containing 10 leaves.
which elongation rate peaked (Fig. 1). Apoplastic peroxidase activity also increased with displacement beyond 40 mm, in the maturation region where secondary cell wall deposition and lignification occur. Specific activity of apoplastic peroxidase was also elevated at the location of maximal elongation rate (Fig. 4, inset), similar to specific activity of peroxidase in the homogenate.

A zymogram of total extractable peroxidase from homogenized leaf blade segments showed that activity of a neutral isofrom was most elevated in the basal 20 mm of the leaf blade (Fig. 6A). A basic (pI 7.6) and acidic (pI 4.6) isofrom were also present in the elongation zone. A new acidic isofrom (pI 5.7) appeared between 15 and 25 mm, which includes the location where epidermal growth ceased. This isofrom appeared to increase in activity as tissue was displaced through the maturation region of the leaf.

A zymogram of extractable peroxidase activity in apoplastic fluid showed highest activity of the neutral and acidic (pI 4.6) isofroms corresponding most closely to the peak of apoplastic peroxidase activity that immediately preceded the drop in cell elongation rate (Fig. 6B). In the segment located 16 to 24 mm distal to the ligule, two acidic peroxidase isofroms appeared with pI 5.6 and 5.7 (Fig. 6B). Based on their extracellular location and high activity from this location through more mature regions of the leaf, these isofroms could function in lignification. Morrison et al. (1994) determined that secondary cell wall deposition and lignification coincided in internodes of maize hybrid Mo17 x B73, and in the leaf blades used in the present study, secondary cell wall deposition followed cessation of elongation, as indicated by increase in insoluble (cell wall) dry weight (Fig. 3).

In tobacco tissues, an anionic cell wall peroxidase with pI 4.2 to 4.5 has been shown to polymerize lignin precursors more rapidly than other isoperoxidases (Mäder et al. 1986). In tall fescue leaf blades, only cationic peroxidases were present during elongation, but two anionic isoperoxidases appeared as elongation stopped and tissue was displaced into the zone of secondary cell wall deposition and lignification (MacAdam et al. 1992b).

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The level of resolution for both developmental and biochemical processes that can be achieved using elongating grass leaf blades allows us to relate peroxidase activity to cell division, elongation and lignification. In conclusion, highest peroxidase activity in the leaf blade homogenate coincides with cell division, while an increase in peroxidase activity in apoplastic fluid is followed by a rapid deceleration of leaf elongation rate. Increased peroxidase activity in the elongation zone is associated with a neutral and an acidic isoform, while new acidic isoforms appear as leaf blade elongation stops and tissue is displaced into the maturation zone, where secondary cell wall deposition and lignification occur. Based on the results of this and previous studies, we propose that peroxidase is secreted into the apoplast at the location of maximal leaf elongation rate, resulting in cell wall enzymatic activity that contributes to the cessation of elongation.

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