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Peroxidase Activity in the Leaf Elongation Zone of Tall Fescue[1]

I. Spatial Distribution of Ionically Bound Peroxidase Activity in Genotypes Differing in Length of the Elongation Zone

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ABSTRACT

Cessation of cell expansion has been associated with cell wall cross-linking reactions catalyzed by peroxidase. This study utilized two genotypes of tall fescue (*Festuca arundinacea* Schreb.) that differ in length of the leaf elongation zone to investigate the relationship between ionically bound peroxidase activity and the spatial distribution of leaf elongation. Peroxidase activity was also localized histochemically in transverse sections of the leaf blade using 3,3′-diaminobenzidine. Soluble or soluble plus ionically bound peroxidase activities were extracted from homogenized segments of the elongating leaf blade and assayed spectrophotometrically. Activity of the ionically bound fraction, expressed per milligram fresh weight or per microgram protein, increased as cells were displaced through the distal half of the elongation zone, corresponding to the region in which the elongation rate declined. In both genotypes, the initial increase in activity preceded the onset of growth deceleration by about 10 hours. In the basal region where elongation began, histochemical localization showed that peroxidase activity was found only in vascular tissues. As cells were displaced farther through the elongation zone, peroxidase activity appeared in walls of other longitudinally continuous tissues such as the epidermis and bundle sheaths. Increase in ionically bound peroxidase activity and changes in localization of peroxidase activity occurred at comparable developmental stages in the two genotypes. The results indicate that cessation of elongation followed an increase in cell wall peroxidase activity.

Leaf area is an important determinant of crop yield and is considered to be more critical than photosynthetic rate (20, 30). An essential factor in improving yield, therefore, is understanding the limitations to leaf expansion. Grasses are a useful experimental system because leaf growth is largely unidirectional, resulting primarily in increase in length. Epidermal cell division is confined to a small region at the leaf base, whereas elongation continues within a defined zone distal to division (17). These factors simplify the quantitative description of growth and its contributing elements (26).

Elongating cells are continually displaced through the elongation zone by the growth of younger cells. Therefore, velocity of displacement of tissue away from the leaf base increases with distance. When spatial growth data are expressed on a temporal basis, the transition from exponential cell elongation to cessation of growth has been shown to occur within a few hours in leaf blades of tall fescue (17) and perennial ryegrass (24). A first step in the evaluation of putative causal agents of growth cessation is to examine their spatial and temporal relationships with the cessation of elongation.

Growth cessation is likely to result from cell wall tightening processes related to formation of cross-linkages among cell wall polymers (3). These cross-linkages include covalent bonds formed following oxidation of phenolic residues of cell wall polysaccharides and structural proteins by peroxidase. Peroxidase may also inhibit elongation by catabolism of IAA, or by oxidation of 1-aminocyclopropane-1-carboxylic acid to ethylene (6). The ionically bound fraction of peroxidase, removable from homogenized tissues with high ionic strength buffers, has been generally accepted to represent cell wall activity, and several studies have reported an association between increase in ionically bound peroxidase activity and the timing or location of cessation of cell expansion (5, 7, 22).

The aim of the present study was to compare the spatial distribution of ionically bound peroxidase activity with the leaf growth profile in two tall fescue (*Festuca arundinacea* Schreb.) genotypes that consistently differ by 25 to 40% in length of the leaf elongation zone. Use of the two genotypes provides a powerful approach to examine whether changes in enzyme activity are related to developmental events. Histochemical localization was used to examine the tissue specificity of peroxidase activity at different positions within the elongation zone. In the companion paper (16), measurements of peroxidase activity in apoplastic fluid extracted from segments of the leaf elongation zone are presented, together with data for the constituent isoforms.

MATERIALS AND METHODS

Plant Material

Plants of tall fescue (*Festuca arundinacea* Schreb.), a perennial C_3 forage grass, were established in a greenhouse from

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vegetative tillers. Tillers of a low-LER[3] or high-LER genotype (10) were transplanted into 15-cm-diameter by 15-cm-deep plastic pots (10 tillers per pot) containing a sphagnum peat moss-vermiculite potting mixture (Terra-Lite Redi-Earth Peat-Lite Mix, W. R. Grace & Co., Cambridge, MA). Pots were watered to the drip-point with deionized water each day and were fertilized daily with 100 mL of Hoagland No. 2 nutrient solution, and weekly with 50 mL of 0.034 M ammonium nitrate and 0.030 M potassium phosphate, and 10 mL of 8.2 mM iron citrate. Greenhouse-grown plants were utilized for histochemistry, and were harvested in October when mean daily air temperature averaged 14°C and natural daylength was approximately 11 h. Plants used for peroxidase assay were transferred to a controlled-environment chamber at a mean photon flux density of 490 μmol m^{-2} s^{-1} PAR at canopy height, a photoperiod of 14 h, constant day/night temperature of 21°C, and RH of 70%. Plants were acclimated to chamber conditions for 6 weeks prior to sampling to ensure that all leaf tissue sampled had initiated and developed under chamber conditions. Plants remained in the vegetative state throughout all experiments.

Growth Measurements

LER in all experiments were calculated as mean daily increase in length over a period of 5 d following emergence of the leaf tip. Earlier work with tall fescue showed that daily growth rates were constant during this stage of leaf development (29). Leaves were sampled for spatial growth analysis, peroxidase activity, and histochemistry 2 d after tip emergence above the whorl, when elongating leaves were less than one-half the length of the next older, fully expanded leaf blade. No sheath elongation had occurred in sampled leaves, but a differentiated ligule, the membrane that occurs on the adaxial surface of the leaf at the junction of the blade and sheath, was present approximately 1 mm above the site of leaf attachment to the apical meristem.

The spatial distribution of cell lengths was determined from replicas of intercostal abaxial epidermal cells. For replicas, location of the ligule was marked by application of 1% (w/v) aqueous acid fuchsin with the tip of a needle, and a thin film of 4% (w/v) Formvar (polyvinyl formaldehyde; Pelco, Tustin, CA) in chloroform was applied with the rounded end of a glass stir rod to the basal 60 mm of the abaxial epidermis. After approximately 20 s, a strip of clear cellophane tape was pressed to the replica, allowing the replica (and acid fuchsin mark) to be lifted away from the leaf and fixed to a glass microscope slide. For greenhouse-grown plants used for histochemistry, replicas of two leaves of each genotype were made at around 0800 h, and cell lengths were calculated from the number of intercostal cells mm^{-1} measured at 1-mm intervals. For chamber-grown plants used for peroxidase assay, replicas of eight leaves of each genotype were made 3 h after the beginning of the light period. Lengths of five intercostal cells were measured at 5-mm intervals beginning 1 mm above the ligule.

The spatial distribution of displacement velocity away from the ligule (mm h^{-1}) was calculated from the LER and cell length profile using the relationship $L_A/L_F = V_A/V_F$, where L_A was the mean cell length at location A within the elongation zone, L_F was the final cell length (mean length of mature cells measured within the 20 mm distal to the elongation zone), V_A was displacement velocity at location A, and V_F was the final displacement velocity (equal to LER) (2, 4, 25, 26). Previous work with tall fescue growing at constant temperature showed that LER and displacement velocities within the elongation zone were higher during the dark period; however, no periodicity of cell lengths reflecting the length of the photoperiod was evident in single columns of epidermal cells within the elongation zone (17). For greenhouse-grown plants, lower dark-period temperatures likely resulted in similar LER and displacement velocities throughout the diurnal cycle. Therefore, the data satisfactorily met the steady-state requirement for application of the above equation (4). To describe the elongation rate profile and define the length of the elongation zone, relative elemental elongation rates (h^{-1}) were calculated from the derivative of a cubic equation fitted to values of V_A plotted against distance above the ligule (27). To convert data from a spatial to a temporal basis, the length of time required for a cell to be displaced over each 5-mm interval within the elongation zone was determined from the calculated displacement velocities.

Peroxidase Assay

Sampling began 3 h after the beginning of a photoperiod. Tillers were excised and older leaves were removed to expose the elongating leaf, which was immediately cooled by placing it on ice. Leaves were cut into sequential 5-mm-long segments that were collected in tared microfuge tubes for determination of fresh weight; mean fresh weight of individual segments ranged from 2.71 mg at the leaf base to 10.94 mg, increasing with distance from the ligule. The leaf segments were then frozen in liquid N and ground with a mortar and pestle, combined with insoluble PVP to immobilize phenolics (13), and mixed with either 0.05 M potassium phosphate buffer (pH 6.0) to extract soluble peroxidase, or with the same buffer containing 0.8 M KCl to extract both soluble and ionically bound ("total") peroxidase (12). One to 1.3 mL buffer was added, depending upon the number of segments, and the proportion of PVP to buffer was 0.08 g PVP mL^{-1}. Homogenized tissue was centrifuged at 16,000*g* for 10 min at 4°C, and the supernatant was assayed for peroxidase activity. Total soluble protein was measured using the Bradford (1) dye method. Each sample consisted of segments from 12 to 25 elongating leaves from a single pot, and three samples were measured for each genotype.

To optimize the peroxidase assay, a range from 0.5 to 10 times the suggested concentration of each substrate (Boehringer Mannheim Biochemica Information II, 1973) was used to determine K_m with peroxidase extracted from mature tall fescue leaf blades. K_m was 0.037 mM for hydrogen peroxide (the H acceptor) and 0.672 mM for guaiacol (the H donor). Final assay concentration of 0.4 mM was approximately 10 times the K_m for hydrogen peroxide, well below the upper limit of 1 mM suggested by Putter and Becker (21). Approximately five times the K_m of guaiacol, or a concentration in

[3] Abbreviations: LER, leaf elongation rate(s); DAB, 3,3'-diaminobenzidine.

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the assay solution of 3 mM, from a 0.022% (w/v) solution in absolute ethanol, gave a linear change in absorbance over time and was used for the final assay solution. A range of pH values for the assay buffer from 3 to 8.5 was tested using acetate/acetic acid, potassium phosphate, and Tris-HCl buffers. The greatest change in absorbance occurred between pH 5.5 and 6.0, the approximate pH of both the vacuole and cell wall (19).

The final peroxidase assay solution was composed of 3.0 mL of 0.1 M potassium phosphate buffer (pH 6.0), 0.04 mL of 0.03 M hydrogen peroxide, and 0.05 mL of 0.2 M guaiacol. The reaction was initiated by adding 0.02 mL extract to the assay solution, which was vortexed and immediately aspirated into the cuvette of a Perkin-Elmer Lambda-3 spectrophotometer equipped with an automated sampling device. Change in A_{436} min^{-1} (Boehringer Mannheim Biochemica Information II, 1973) increased linearly with enzyme concentration using dilution series of both a purified horseradish peroxidase (Sigma P-8250) and an extract from mature tall fescue leaf blades.

Histochemistry

The basal 50 mm of elongating leaf blades was excised, fixed in 2.5% (w/v) glutaraldehyde (0.1 M potassium phosphate buffer, pH 7.0, 4°C) for 30 min (8), and rinsed twice for 15 min in the same buffer, and a third time in 0.05 M Tris-HCl buffer (pH 5.0, 4°C). One-millimeter-long segments at 5-mm intervals within the elongation zone were excised and incubated for 15 min at room temperature in 0.05% (w/v) DAB and 0.01% (w/v) hydrogen peroxide (0.05 M Tris-HCl buffer, pH 5.0) (9). Control segments were incubated in DAB without hydrogen peroxide. Oxidation of DAB by peroxidase in the presence of hydrogen peroxide resulted in an insoluble brown osmiophilic product, identifying the location of peroxidase in the tissue.

After rinsing twice for 15 min in 0.05 M Tris-HCl buffer (pH 5.0, 4°C) and once in 0.1 M potassium phosphate buffer (pH 7.0, 4°C), segments were postfixed in 1% (w/v) osmium tetroxide (0.1 M potassium phosphate buffer, pH 7.0, 4°C) for 2 h, rinsed three times for 30 min in the same buffer, and dehydrated in an ethanol series (30 min per step). Segments were then infiltrated with a graded Spurr's (28) series using a rotary mixer for 0.5 d at each concentration (1:3, 1:1, 3:1, 1:0; Spurr's:absolute ethanol), and embedded in Spurr's. Blocks were trimmed and 3-μm-thick transverse sections were made using an LKB Ultrotome.

RESULTS

Spatial Distribution of Leaf Growth and Peroxidase Activity

LER of plants used for peroxidase assay were 29 ± 4.8 and 34 ± 3.2 mm d^{-1} for the low-LER and high-LER genotypes, respectively (± SD, $n = 9$). Length of the elongation zone of the low-LER genotype was 25 to 30 mm, with the maximum relative elemental elongation rate occurring between 10 and 15 mm above the ligule (Fig. 1A). In the high-LER genotype, length of the elongation zone was 35 to 40 mm, and the maximum relative elemental elongation rate occurred between 15 and 20 mm above the ligule (Fig. 1B). Cell length data from which the elongation rate profiles were derived are shown in the inset of each figure.

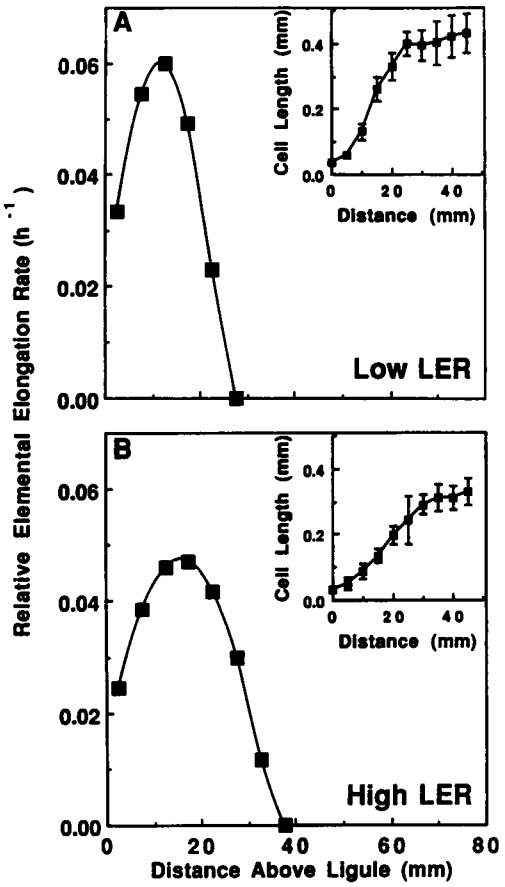

Figure 1. Spatial distribution of relative elemental elongation rates in leaf blades of low-LER (A) and high-LER (B) genotypes of tall fescue. Data were calculated using the first derivative of an equation for displacement velocity against distance above the ligule, and displacement velocities were derived from means of epidermal cell lengths as described in the text. The insets show intercostal abaxial epidermal cell lengths in leaf blades of low-LER (A) and high-LER (B) genotypes of tall fescue. Error bars at each location represent ± SE of means from eight leaves.

Peroxidase activity was expressed as a function of tissue fresh weight rather than as activity per mm leaf length to facilitate comparison of the two genotypes, because the low-LER genotype has narrower leaves and lower fresh weight per mm length than the high-LER genotype (14). In both genotypes, activities of soluble and total peroxidase per mg fresh weight were highest near the ligule, and decreased with distance over the basal one-third of the elongation zone (Fig. 2). Activity of soluble peroxidase continued to decrease as tissue was displaced farther through the elongation zone, whereas total peroxidase activity remained relatively constant. Values of soluble and total peroxidase activity were equivalent for the two genotypes at the distal end of the elongation zone: ΔA_{436} min^{-1} mg^{-1} fresh weight was approximately 4 for soluble activity and 7 for total activity. Soluble and total activities differed significantly from each other distal

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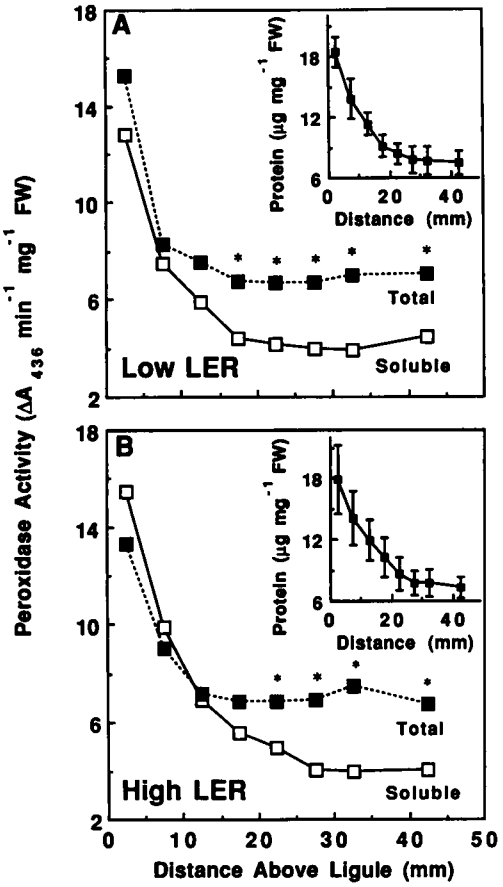

Figure 2. Peroxidase activity per mg fresh weight in leaf blades of low-LER (A) and high-LER (B) genotypes of tall fescue. Peroxidase was extracted from 5-mm-long leaf blade segments by homogenization in 0.05 M potassium phosphate buffer (soluble) or in the same buffer plus 0.8 M KCl (total). The ionically bound fraction was the difference between total and soluble activities. Data are means of three replications. LSD were calculated; $P \leq 0.05$ indicated by *. The insets show total soluble protein per mg fresh weight in leaf blades of low-LER (A) and high-LER (B) genotypes of tall fescue. Error bars represent ± SD ($n = 6$).

to 15 mm above the ligule in the low-LER and 20 mm in the high-LER genotype. Therefore, activity of ionically bound peroxidase (the difference between the total and soluble fractions) increased with displacement through the distal half of the elongation zone in each genotype, corresponding to the region in which relative elemental elongation rate declined (Fig. 1).

The increase in ionically bound peroxidase activity occurred despite a progressive decrease in total soluble protein per mg fresh weight as tissue was displaced through the elongation zone (Fig. 2, insets). Accordingly, when ionically bound peroxidase activity was expressed on a unit protein or specific activity basis, a distinct increase occurred in the distal half of the elongation zone. These data are shown in Figure 3 on a temporal basis to illustrate change in peroxidase activity and relative elemental elongation rate associated with a particular tissue element as it was displaced through the elongation zone over time. In each genotype, increase in the specific activity of ionically bound peroxidase began about 10 h before the relative elemental elongation rate began to decline. As peroxidase activity continued to increase over the succeeding 20 h, elongation rate decreased to zero. Thus, despite the difference in length of the elongation zone between the two genotypes, there was a consistent correlation between increase in activity of ionically bound peroxidase and decline of relative elemental elongation rate.

Histochemical Localization of Peroxidase Activity

Histochemical localization in transverse sections from the leaf elongation zone was used to determine the tissue specificity of peroxidase activity. Leaves of greenhouse-grown plants used for this study grew more slowly than those of the chamber-grown plants used for the peroxidase assay because of the shorter photoperiod and cooler night temperatures in the greenhouse: LER were 8 ± 1.5 and 14 ± 1.6 mm d^{-1} for the low-LER and high-LER genotypes, respectively (± SD, $n = 6$). Elongation zone lengths were also somewhat

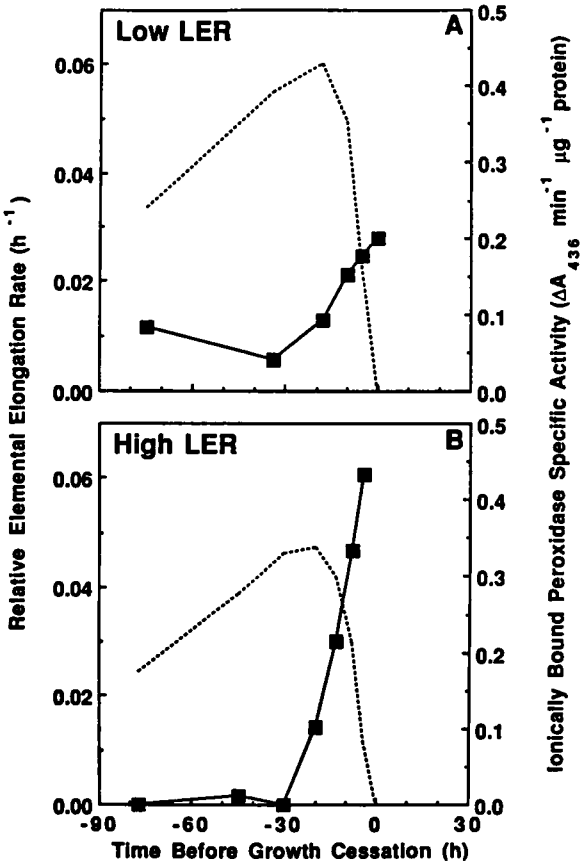

Figure 3. Ionically bound peroxidase activity per μg protein (solid lines) and relative elemental elongation rates (dashed lines, from Fig. 1) in leaf blades of low-LER (A) and high-LER (B) genotypes of tall fescue plotted against time before cessation of growth. Data illustrate values associated with a particular tissue element as it was displaced through the elongation zone. Specific activity of ionically bound peroxidase was calculated as the difference between specific activities of total and soluble peroxidase.

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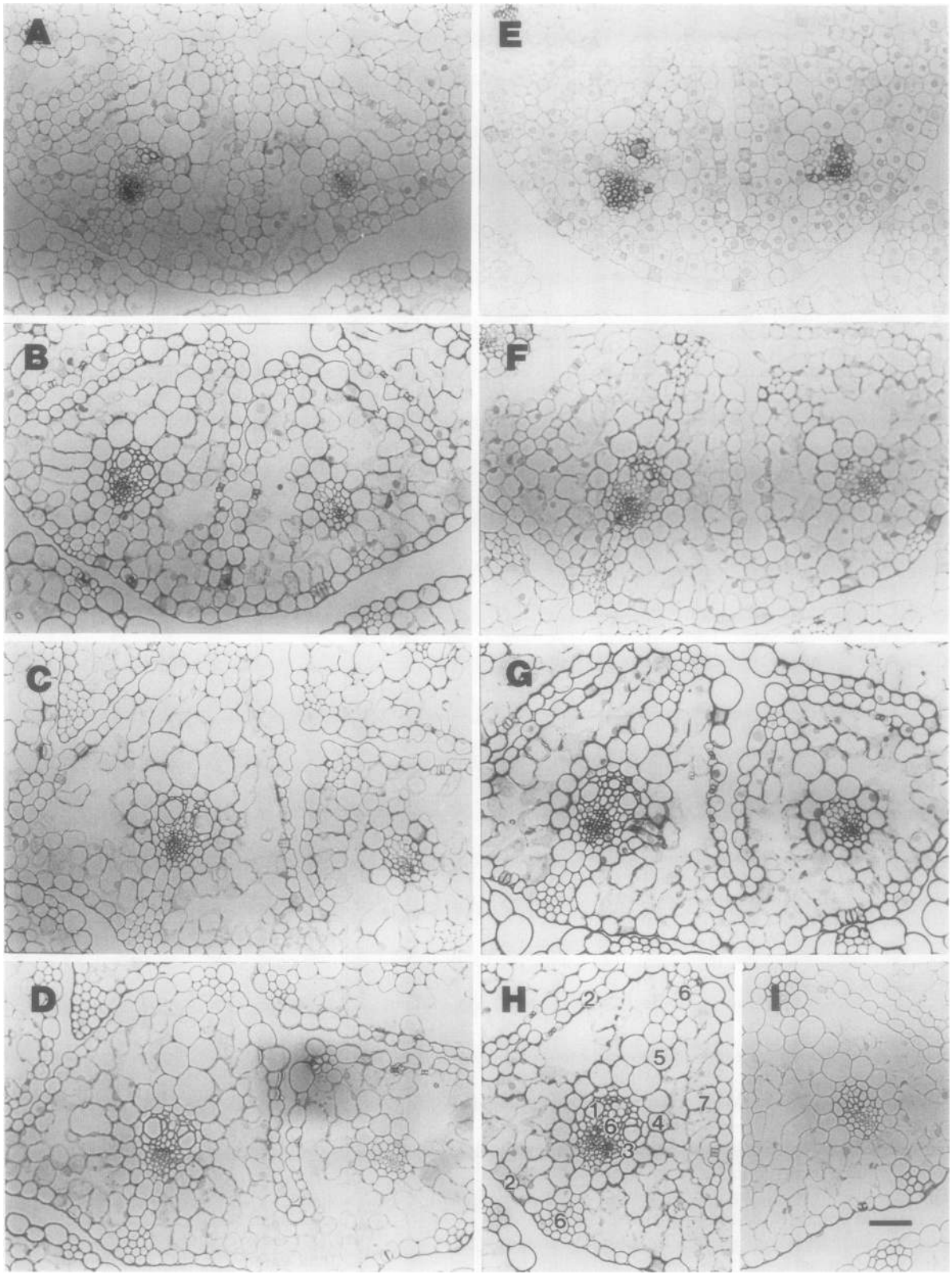

Figure 4. Localization of peroxidase in major and minor ridges in leaf blades of the low-LER genotype of tall fescue at 5 (A), 15 (B), 25 (C), and 35 (D) mm above the ligule, and of the high-LER genotype at 5 (E), 15 (F), 25 (G), and 35 (H) mm above the ligule. Length of the elongation zone was 15 to 20 mm for the low-LER and 20 to 25 mm for the high-LER genotype. One-millimeter-long segments of the elongating leaf blade were incubated in a 0.05% solution of DAB and 0.01% hydrogen peroxide for 15 min. Segments were postfixed with osmium tetroxide, embedded in Spurr's, and sectioned 3 μm thick. Peroxidase activity is indicated by a dense precipitate, seen primarily in cell walls. Control segments were incubated in DAB without the addition of hydrogen peroxide. The absence of staining in the control section at 35 mm above the ligule of the high-LER genotype is shown in (I). Cell types are labeled in (H): 1, xylem; 2, epidermis; 3, inner bundle sheath; 4, outer bundle sheath; 5, bundle sheath extension; 6, fiber bundle; 7, mesophyll. Bar = 50 μm for all sections.

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less: 15 to 20 mm for the low-LER and 20 to 25 mm for the high-LER genotype.

At 5 mm above the ligule in the high-LER genotype (Fig. 4E), in the earliest stages of leaf elongation, peroxidase activity was localized in the protoxylem and phloem tissues. Activity at this location was similar in major ridges (containing vascular bundles with xylem) and adjacent minor ridges (vascular bundles without xylem). All sections displayed faint background staining caused by fixation with osmium tetroxide. In the low-LER genotype, localization of peroxidase activity at 5 mm above the ligule (Fig. 4A) was seen not only in the vascular tissue, but also in cell wall junctions in the bundle sheaths and epidermis, similar to that at 15 mm above the ligule in the high-LER genotype (Fig. 4F). Thus, differences between genotypes in localization of peroxidase activity reflected differences in the location at which successive stages of tissue differentiation occurred.

At 25 mm above the ligule in the high-LER genotype (Fig. 4G), near the distal end of the elongation zone of this genotype, staining of cell walls in longitudinally continuous tissues was more intense than at 15 mm (Fig. 4F). The tissues that expand longitudinally include the abaxial and adaxial epidermis, inner and outer bundle sheaths and bundle sheath extensions, and the abaxial, adaxial, and vascular fiber bundles. Staining was also apparent in mesophyll cells, which are oriented radially between the vascular tissue and the epidermis. At 15 mm above the ligule in the low-LER genotype (Fig. 4B) and 25 mm above the ligule in the high-LER genotype (Fig. 4G), peroxidase activity was seen throughout the walls of the epidermis and bundle sheaths. At 25 mm above the ligule in the low-LER genotype (Fig. 4C) and 35 mm above the ligule in the high-LER genotype (Fig. 4H), within the region of secondary cell wall deposition (17), staining intensity was somewhat reduced compared with the sections at 15 (Fig. 4B) and 25 mm (Fig. 4G), respectively. Staining intensity was further reduced in the low-LER genotype at 35 mm above the ligule (Fig. 4D). A control section from 35 mm above the ligule of the high-LER genotype (Fig. 4I) illustrates the appearance of tissue incubated in DAB without the addition of hydrogen peroxide. As in the growth zones of pea roots and leaves (9), staining for peroxidase activity in the elongation zone occurred primarily in the middle lamella and in cell walls; little protoplasmic activity was seen except in vascular tissue.

DISCUSSION

Both the quantitative and histochemical data indicated that an increase in activity of the peroxidase fraction associated with the cell wall occurred prior to cessation of elongation in tall fescue leaf blades. Increase in activity of ionically bound peroxidase began after cells had been displaced about one-third of the distance through the elongation zone, or about 30 h before growth cessation, in a location where relative elemental elongation rate was still increasing. Elongation rates peaked soon thereafter, and as ionically bound peroxidase activity continued to increase, elongation slowed and stopped. This correlation between increase in peroxidase activity and cessation of elongation was consistent for both genotypes, although they differed in elongation zone length, indicating that increase in peroxidase activity was related to stage of development rather than location.

In other experimental systems, ionically bound peroxidase activity also increased prior to cessation of elongation. In cotton fibers, elongation occurred over a period of 30 d; activity of ionically bound peroxidase increased from day 15 to day 30, whereas soluble peroxidase activity remained nearly constant (22). Fiber elongation rate declined as ionically bound peroxidase activity increased. In etiolated mung bean hypocotyls divided into three segments of decreasing growth rate with distance from the hook, an increase in ionically bound peroxidase activity occurred with decreasing growth rate, whereas activity of the soluble fraction decreased with distance from the hook (7).

In transverse sections of the tall fescue elongation zone stained for enzyme activity, peroxidase was limited to the vascular tissue in the earliest stages of elongation. Protoxylem differentiates earlier than other tissues and is functional in the elongation zone (14). Other longitudinally expanding tissues such as the epidermis and bundle sheaths differentiate later, and peroxidase activity appeared in these tissues farther from the ligule. Goldberg *et al.* (7) found that peroxidase activity in walls of the epidermis and outer layers of cortical parenchyma cells in mung bean hypocotyls increased as cells vacuolated. It has been suggested that the outer tissue layers may determine the rate of expansion of an entire organ (11); our histochemical data demonstrate that peroxidase activity appeared in epidermal and bundle sheath cell walls as relative elemental elongation rates peaked and began to decline.

Although there is biosynthesis of cell wall material in the elongation zone (23), the content of cell wall material per mm leaf length within the elongation zone decreases with distance above the ligule (15). This is because the number of cross walls per mm decreases as cells elongate, and it also appears that cell walls thin as elongation progresses (14). Cell wall content and total soluble protein content decrease similarly with distance above the ligule (14); therefore, the activity of ionically bound peroxidase per unit cell wall would be expected to increase in the same way that specific activity of ionically bound peroxidase increased. Increase in the specific activity of ionically bound peroxidase is noteworthy because other proteins, such as the structural protein extensin (which is initially soluble), are likely to be synthesized in large quantities in the same region.

Ionically bound peroxidase has been equated with the cell wall fraction because it is the activity that remains associated with cell walls of homogenized tissue until the tissue is extracted with high ionic strength buffers. Soluble peroxidase has been thought to represent the cytosolic or vacuolar fractions. Only the peroxidase activity actually located in cell walls of intact tissue would be relevant to cell wall cross-linking, however, and the *in vivo* location of ionically bound isozymes of peroxidase has recently been questioned. Mader *et al.* (18) demonstrated that vacuolar peroxidases of suspension-cultured tobacco cells became ionically bound to cell walls when cells were homogenized, suggesting that in any system, some of the ionically bound peroxidase activity extracted after homogenization may be artifactually associated with cell walls. Therefore, to test further the hypothesis that an increase in cell wall peroxidase activity is associated with

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cessation of elongation, we present data in the companion paper (16) for peroxidase activity in apoplastic fluid extracted from segments of the leaf elongation zone in the same two tall fescue genotypes.

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