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# Tissue-Specific Expression of Germin-Like Oxalate Oxidase during Development and Fungal Infection of Barley Seedlings[1]

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**Oxalate oxidase activity was detected in situ during the development of barley seedlings. The presence of germin-like oxalate oxidase was confirmed by immunoblotting using an antibody directed against wheat germin produced in *Escherichia coli*, which is shown to cross-react with barley (*Hordeum vulgare*) oxalate oxidase and by enzymatic assay after electrophoresis of the protein extracts on polyacrylamide gels. In 3-d-old barley seedlings, oxalate oxidase is localized in the epidermal cells of the mature region of primary roots and in the coleorhiza. After 10 d of growth, the activity is detectable only in the coleorhiza. Moreover, we show that oxalate oxidase is induced in barley leaves during infection by the fungus *Erysiphe graminis* f. sp. *hordei* but not by wounding. Thus, oxalate oxidase is a new class of proteins that responds to pathogen attack. We propose that oxalate oxidase could have a role in plant defense through the production of $H_2O_2$.**

Oxalate oxidase (oxalate:oxygen oxidoreductase, EC 1.2.3.4), an enzyme that degrades oxalate to $CO_2$ and $H_2O_2$, has been characterized in some plant species including barley (*Hordeum vulgare*) (Sugiura et al., 1979; Pietta et al., 1982), but the specific biochemical involvements of the activity remain to be established. It has recently been shown that wheat (*Triticum aestivum*) and barley oxalate oxidase belong to the germin family of proteins, for which a possible (oxidative) role in plant defense was first mentioned many years ago (Lane et al., 1986). The recent demonstration of an oxalate oxidase activity for wheat germin (Dumas et al., 1993; Lane et al., 1993) immediately suggested specific molecular mechanisms by which the germins might participate in plant defense and development (see Lane, 1994).

Germins are glycoproteins detected in monocotyledons such as wheat, oat, rye, and barley (Grzelczak et al., 1985), whose synthesis is induced during germination of wheat embryos (Grzelczak and Lane, 1984). Germin oligomers are refractory to dissociation during SDS-PAGE and to digestion by proteases (Grzelczak and Lane, 1984). In barley we have shown that the accumulation of germin during germination paralleled the induction of oxalate oxidase activity (Dumas et al., 1993). Although germin was initially discovered in cereals by Lane and co-workers, a cDNA sequence showing a significant homology with germin has been isolated from *Arabidopsis thaliana* (Höfte et al., 1993; Delseny et al., 1994), suggesting that germin homologs exist in dicotyledons. Recently, two polypeptides, Gs1 and Gs2, induced during salt stress of a salt-tolerant barley cultivar, were identified as germin-like proteins by amino acid sequencing (Hurkman et al., 1991, 1994), suggesting that members of the germin family may also have a protective function during osmotic stress. Moreover, studies of changes of protein synthesis during salt stress of the halophyte *Mesembryanthemum crystallinum* led to the cloning of a cDNA induced by salt stress that has sequence similarity to germin (Michalowski and Bohnert, 1992; Andolfatto et al., 1994). However, it remains to be verified if these different germin homologs are oxalate oxidases. For example, Lane (1994) mentioned that spherulins 1a/1b from *Physarum polycephalum*, which are known to be related at the amino acid sequence level to wheat germin (Lane et al., 1991), do not have oxalate oxidase activity.

By taking advantage of a rapid procedure to detect oxalate oxidase activity in plant tissues, based on the singular properties of germin-like oxalate oxidase, we have localized this enzyme at different stages of barley seedling growth and during infection of barley leaves by the fungus *Erysiphe graminis* f. sp. *hordei*. We show a good correlation between oxalate oxidase activity and the presence of germin as detected with antibodies raised against a nonglycosylated wheat germin monomer produced in *Escherichia coli*.

## MATERIALS AND METHODS

### Plant Material

Barley (*Hordeum vulgare* L.) without any known powdery mildew resistance gene was used for the experiments. Seeds were allowed to germinate on Whatman No. 3 filter paper that had been soaked in water or grown for 10 d in pots (10 plants/pot). Infection was achieved by blowing conidia from plants heavily infected by *Erysiphe graminis* f. sp. *hordei* onto uninfected plants. For wounding experiments leaves were injured by cutting about 20 parallel incisions, 5 mm apart, with a razor blade.

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### Preparation of the Protein Extracts

Plant tissues (0.2 g) were ground in liquid nitrogen and resuspended in 1 mL of water containing 0.1% SDS. After centrifugation (10,000*g* for 15 min), proteins present in the supernatant were analyzed by gel electrophoresis. Polypeptides from about 10 mg of tissue (50 μL of extract) were loaded onto polyacrylamide gels.

### Production of Antibodies Directed Against the Wheat Germin Produced in *Escherichia coli*

The sequence coding for wheat mature germin (isoform G; Dratewka-Kos et al., 1989; Lane et al., 1991) was cloned in the pMAL-cRI vector (New England Biolabs, Beverly, MA) in the same translational reading frame as the *malE* gene, which encodes the maltose-binding protein (Dumas et al., 1993). After induction with IPTG, a protein extract was prepared by sonication of a 400-mL culture and the maltose-binding protein–germin fusion protein was purified by affinity chromatography on an amylose column (New England Biolabs) according to the manufacturer's instructions. The purified fusion protein (100 μg) was administered to a rabbit in four intramuscular injections. Ten days after the last boost, the serum was collected, clarified by centrifugation, and stored at −20°C.

### PAGE and Immunoblotting

Proteins were analyzed by polyacrylamide slab gel electrophoresis according to Laemmli (1970). For immunoblotting the basic procedure of Towbin et al. (1979) was used. For immunodetection anti-germin serum was diluted 1:2000, anti-tobacco chitinase (Legrand et al., 1987) was diluted 1:2000, and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:2000. The blots were stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$.

### Enzyme Assays

For detection of oxalate oxidase activity with gel electrophoresis, the proteins were loaded without heat denaturation onto an SDS polyacrylamide gel (10%) and transferred onto a nitrocellulose sheet that was then directly incubated with oxalic acid (2.5 mM) in succinate buffer, pH 4 (25 mM succinic acid, 3.5 mM EDTA), containing 1 unit $mL^{-1}$ horseradish peroxidase and 4-chloro-1-naphthol (0.6 mg $mL^{-1}$) as staining reagent. For the localization of oxalate oxidase, plant tissues or tissue sections were directly incubated in this solution without peroxidases. No staining appeared when oxalic acid was omitted in the staining solution.

### Inhibition of Oxalate Oxidase Activity by Antibodies Raised Against Wheat Germin Produced in *E. coli*

Five to 10 μL of immune serum or preimmune serum were mixed with 200 μL of protein A-Sepharose (30 mg $mL^{-1}$) in TBS-Tween (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20). The protein A-Sepharose was washed three times with TBS-Tween and 20 ng of purified barley oxalate oxidase (Dumas et al., 1993) were added. After 1 h of incubation oxalate oxidase activity was measured in the supernatant following the method of Sugiura et al. (1979).

## RESULTS

### Location of Oxalate Oxidase Activity during Development of Barley Seedlings

The location of oxalate oxidase was achieved by incubating the tissues in a buffer containing oxalic acid and the chromogenic substrate 4-chloro-1-naphthol. The degradation of oxalic acid by oxalate oxidase produces $H_2O_2$, which is used by endogenous peroxidases to oxidize 4-chloro-1-naphthol, causing the formation of a precipitate that turns dark blue. Since staining occurs only in the presence of oxalic acid, this method allows a rapid and specific localization of oxalate oxidase activity.

Barley seeds were allowed to germinate and oxalate oxidase activity was detected as described in "Materials and Methods" after 3 d of germination. Strong staining appeared rapidly over the mature regions of primary roots and the coleorhiza, whereas tips, elongating areas, and the coleoptile remained clear (Fig. 1A). The staining of a cross-section of the root showed that this activity is localized mainly in the root epidermis (Fig. 1B). Ten-day-old seedlings were also analyzed in the same way; a high oxalate oxidase activity was confined to the coleorhiza (Fig. 1C).

The proteins of the different parts of the seedlings were extracted and oxalate oxidase activity was detected with gel electrophoresis and blotting of the proteins (Dumas et al., 1993). After 3 d of germination an oxalate oxidase activity was detected only in root extracts (Fig. 2A). However, after 10 d of growth the activity disappeared from the root and was found only in the coleorhiza extract (Fig. 2B).

To obtain a specific antiserum against wheat germin, antibodies were raised in a rabbit by injecting purified wheat germin produced in *E. coli* (Dumas et al., 1993). As shown in Figure 3, these antibodies, diluted 2000-fold, strongly recognized the monomer of the purified barley oxalate oxidase. Moreover, a single band was detected from protein extracts prepared from germinating barley embryos without background, due to antibodies that react with carbohydrate moieties (Fig. 3, lanes 2 and 3). The oligomeric oxalate oxidase was poorly recognized by the antibodies raised against the germin monomer produced in *E. coli* (data not shown) according to the results reported by Lane et al. (1992). However, by using the antiserum without dilution, the barley oxalate oxidase activity can be removed in vitro by adding these germin antibodies bounded to protein A-Sepharose prior to the oxalate oxidase assay. Undiluted antibodies (5–10 μL) bound to protein A-Sepharose were mixed with 20 ng of purified barley oxalate oxidase, resulting in a decrease of 72 and 78%, respectively, of the oxalate oxidase activity. No removal of the activity was observed by mixing 10 μL of preimmune serum with the purified barley oxalate oxidase prior to the oxalate oxidase assay. These results confirm that wheat

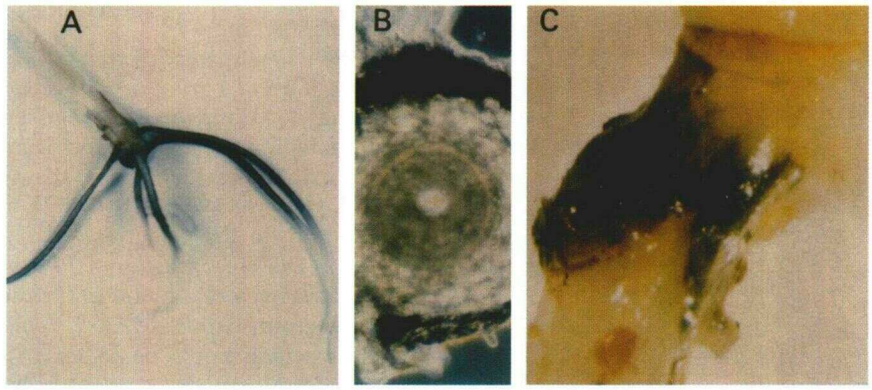


**Figure 1.** Localization of oxalate oxidase activity during development of barley seedling. A, Barley seedling stained for oxalate oxidase activity after 3 d of growth as described in "Materials and Methods." The blue color indicates the presence of activity. B, Hand section of a 10-d-old barley seedling root was stained for oxalate oxidase activity and examined under a light microscope (magnification ×75). C, Localization of oxalate oxidase activity in 10-d-old barley seedling.

germin (isoform G) and the barley oxalate oxidase share common epitopes (Dumas et al., 1993; Lane et al., 1993) and that the cross-reaction is not due to the carbohydrate moiety of the proteins, since the antibodies were directed against germin apoprotein produced in *E. coli*.

Protein extracts from 3- and 10-d-old barley seedlings were separated by electrophoresis on SDS polyacrylamide gels and subjected to immunoblot analysis using antibodies raised against the bacterially expressed wheat-germin apoprotein. Two germin isoforms were detected in the root extract from 3-d-old plants (Fig. 4A). After 10 d of growth, these isoforms were found only in the coleorhiza extract (Fig. 4B). In these plants, germin antibodies allowed the detection of a protein in the coleoptile extract (Fig. 4B).

### Induction of Oxalate Oxidase upon Infection by *E. graminis* f. sp. *hordei*

The induction of oxalate oxidase was analyzed during the infection of barley by a phytopathogenic fungus, *E. graminis* f. sp. *hordei*. During this interaction a number of defense-related genes were induced, even in the case of a compatible host-pathogen interaction (Boyd et al., 1994; Ignatius et al., 1994).

Five days after infection, staining of an infected leaf for oxalate oxidase activity showed that activity was induced during the infection and was localized mainly along the vascular bundles (Fig. 5). Leaf proteins were extracted and analyzed for oxalate oxidase activity 3 and 5 d after the infection. Between 3 and 5 d after infection, a discrete induction of germin-like oxalate oxidase activity (Fig. 6A) coincided with a discrete induction of serologically detectable amounts of the germin apoprotein (Fig. 6B). To compare the pattern of expression of the oxalate oxidase with a defense-related protein, antiserum directed against a tobacco chitinase (Legrand et al., 1987) was used to detect barley chitinase in healthy and infected leaves. During the

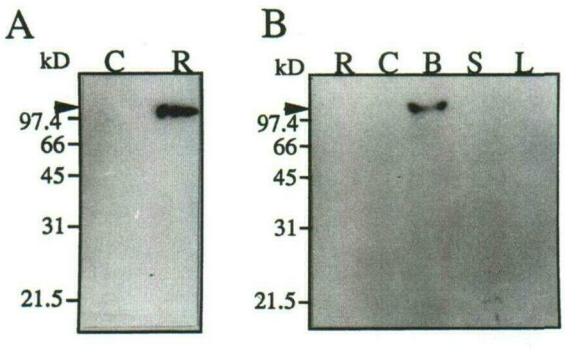


**Figure 2.** Detection of oxalate oxidase activity in barley tissues. Proteins extracted from 3-d-old plants (A, lane C, coleoptile; lane R, roots) and 10-d-old plants (B, lane R, roots; lane C, coleoptile; lane B, coleorhiza; lane S, stem; lane L, leaf) were separated by electrophoresis on 10% SDS polyacrylamide gels without heat denaturation. Oxalate oxidase activity was revealed upon transfer of the protein onto nitrocellulose as described by Dumas et al. (1993). The position of oxalate oxidase is indicated by the arrowheads, corresponding to a molecular mass of about 120 kD.

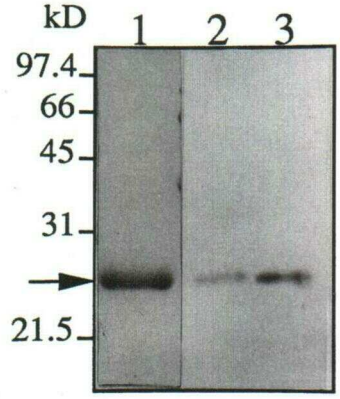


**Figure 3.** Characterization of antibodies raised against the wheat germin produced in *E. coli*. Proteins were analyzed by immunoblotting after heat denaturation of the samples using an antiserum directed against the wheat germin produced in *E. coli*. Ten nanograms of purified barley oxalate oxidase (lane 1) or 10 μg of total protein extract from 48 and 72 h (lanes 2 and 3, respectively) postimbibition of barley embryos were loaded onto a 12% SDS polyacrylamide gel. The position of oxalate oxidase monomer is indicated by the arrow.

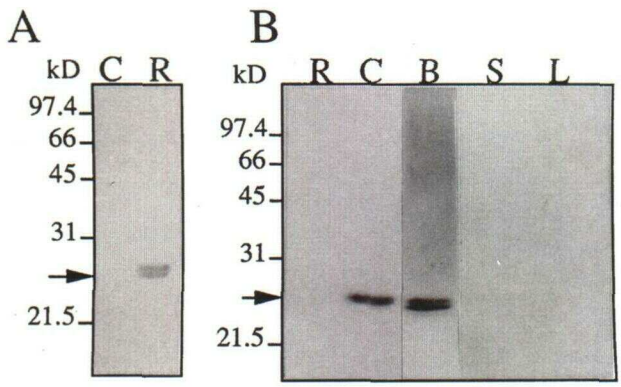


**Figure 4.** Analysis by immunoblotting of barley extracts with antibodies directed against wheat germin produced in *E. coli*. Proteins extracted from 3-d-old plants (A, lane C, coleoptile; lane R, roots) and 10-d-old plants (B, lane R, roots; lane C, coleoptile; lane B, coleorhiza; lane S, stem; lane L, leaf) were loaded onto a 12% SDS polyacrylamide gel. The position of oxalate oxidase is indicated by the arrows.

infection, the induction of the oxalate oxidase paralleled the accumulation of a 25-kD chitinase (Fig. 6), which was recently shown to be induced by powdery mildew (Boyd et al., 1994; Ignatius et al., 1994). As for oxalate oxidase, a constitutive expression of chitinase was observed in the coleorhiza and to a lesser extent in the coleoptile from the 10-d-old healthy plant (Fig. 6C).

In contrast to chitinase (Ignatius et al., 1994), wounding of healthy leaves did not induce oxalate oxidase. In the case of wounding, oxalate oxidase activity was not detectable, nor did immunoblot analysis detect an accumulation of the oxalate oxidase protein (data not shown).

## DISCUSSION

In this paper we describe the localization of oxalate oxidase during barley seedling development. In young seedlings (3 d of growth) the enzyme is localized in the mature region of the root epidermis as well as in the coleorhiza, in accordance with the work of Hurkman et al. (1991). However, after 10 d of growth the activity was found only in the coleorhiza and was not detectable in roots. In the coleoptile from 10-d-old seedlings, a 26-kD protein was detected by the antibodies directed against the wheat germin expressed in *E. coli*. However, no oxalate oxidase activity could be detected. This suggests that germin isoforms can differ in their ability to degrade oxalic acid or that an inactive form of oxalate oxidase can accumulate in this tissue.

It has been shown that several germin isoforms are induced during the development and germination of wheat embryos (Grzelczak and Lane, 1984; Grzelczak et al., 1985; Lane et al., 1987, 1992), as well as in roots of salt-stressed barley plants (Hurkman et al., 1991, 1994). Since only the wheat germin isoform G has been shown to be an oxalate oxidase (Lane et al., 1993), it remains unclear whether all these germin isoforms possess oxalate oxidase activity. Until now the accumulation of germin was correlated with an increase of oxalate oxidase activity only during the germination of barley grains (Dumas et al., 1993) and not during salt stress. The purification to homogeneity of the germins from the different tissues is necessary to determine the enzymatic activity of these isoforms.

Five days after powdery mildew infection, the induction of oxalate oxidase activity corresponding to an accumulation of a germin isoform was observed in infected leaf tissue, suggesting that oxalate oxidase can also play a role during the plant defense response. The constitutive expres-

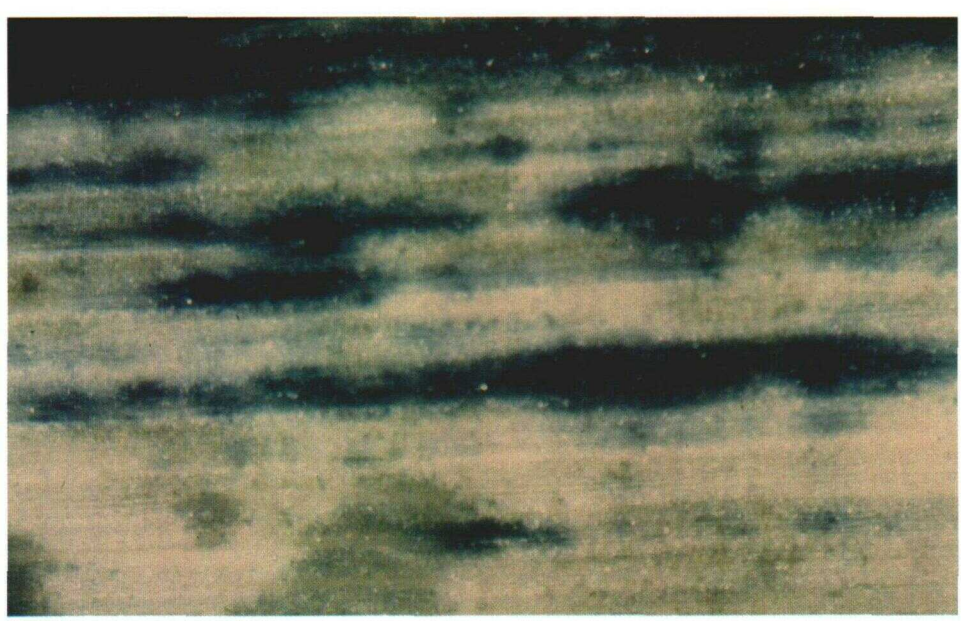

**Figure 5.** Localization of oxalate oxidase activity in barley leaf 5 d after inoculation with *E. graminis* f. sp. *hordei*.

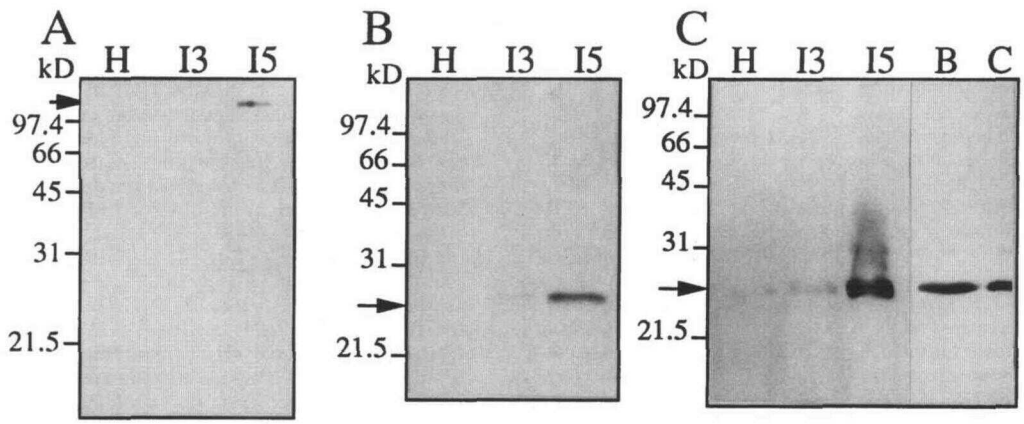


**Figure 6.** Accumulation of oxalate oxidase in barley leaves infected by *E. graminis* f. sp. *hordei*. A, Proteins extracted from healthy leaves (lanes H) and leaves infected for 3 d (lanes I3) and 5 d (lanes I5) were separated by electrophoresis on a 10% SDS polyacrylamide gel without heat denaturation and transferred onto nitrocellulose. Oxalate oxidase activity was revealed according to Dumas et al. (1993). B and C, The same protein extracts were subjected to immunoblot analysis using antibodies directed against wheat germin produced in *E. coli* (B) or antibodies directed against a tobacco chitinase, PR Q (C). The lanes B and C in panel C correspond to healthy plant extracts from either the coleorhiza or coleoptile. The arrows indicate the location of oxalate oxidase holoenzyme (A), oxalate oxidase monomer (B), or chitinase (C).

sion of oxalate oxidase and chitinase in the coleorhiza may create a difficult environment for pathogens to grow near this tissue, which is in contact with many soil microorganisms. Interestingly, another defense-related protein is also expressed during germination: the PRms is a pathogenesis-related protein that accumulates in the scutellum of germinating maize seeds (Casacuberta et al., 1991; Cordero et al., 1992). Also, it has been shown that a different stress-related protein (osmotin) not only accumulates in NaCl and desiccation-adapted tobacco cells but also during fungal and virus infection in tomato and tobacco (Stintzi et al., 1991; Woloshuk et al., 1991). Thus, as for other pathogenesis-related proteins, oxalate oxidase is induced under various conditions of stress (osmotic stress and infection), as well as during natural processes such as germination. It remains to be elucidated what could be the function of oxalate oxidase activity during these different situations.

Recently, Lane (1994) proposed that germin plays a role in the remodeling of the extracellular matrix of ground tissues through the incorporation of highly substituted glucuronogalactoarabinoxylans. This hypothesis is supported by the selective association between germin and the highly substituted glucuronogalactoarabinoxylans (Lane et al., 1987, 1992) and the production of $H_2O_2$ required for cross-linking reactions in the cell wall (Fry, 1986).

One of the reaction products formed by oxalate oxidase is $H_2O_2$, and the rapid release of $H_2O_2$ has been reported to occur in several plants upon treatment with an elicitor of plant defense responses (Apostol et al., 1989; Devlin and Gustine, 1992; Nürnberger et al., 1994). Moreover, Chen et al. (1993) recently demonstrated that salicylic acid, a natural signal molecule for the activation of plant defense responses, inhibits a catalase isoenzyme in tobacco leaves, leading to increased levels of $H_2O_2$. Because injected $H_2O_2$ as well as chemicals that increase $H_2O_2$ levels led to the induction of plant defense genes (Chen et al., 1993), this result supports an important role of $H_2O_2$ in the development of the defense response. Thus, degradation of oxalate by oxalate oxidase could lead to an enhancement of the defense response through the production of $H_2O_2$. On the other hand, $H_2O_2$ produced by the degradation of oxalic acid by oxalate oxidase can be used as a substrate by peroxidases that are induced during the development of powdery mildew in wheat and barley (Schweizer et al., 1989; Thordal-Christensen et al., 1992; Boyd et al., 1994). In this case, the induction of oxalate oxidase activity can contribute to the ability of the infected tissue to lignify and to the formation of cell wall appositions (papillae) (Gold et al., 1986).

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