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Abstract : Oxalic acid or more commonly its salts, oxalates, are widely distributed throughout the plant kingdom. Oxalic acid is generally considered an inert product of metabolism and its accumulation is toxic to tissues. Therefore, the enzymes degrading oxalic acid have received considerable attention. Oxalate oxidase is an oxidoreductase which degrades oxalate into CO_2 and H_2O_2. Recently, oxalate oxidase was shown to have high amino acid homology with germin proteins and at the same time cereal germin proteins were shown to have oxalate oxidase activity.

Germin gene expression was shown to be auxin responsive. It was well demonstrated that coleoptile cells underwent extensive elongation upon auxin treatment. In the current study, it was shown that during auxin-induced wheat coleoptile elongation there was an increase in the activity of germin-like oxalate oxidase. This activity which produce H_2O_2 may restrict cell elongation by mediating cross-linking of cell wall polymers.

Key Words: Auxin, Coleoptile elongation, Germin-like oxalate oxidase

Oksin Germin-benzeri Okzalat Oksidaz Aktivitesini Buğday Koleoptillerinde Arttırır

Özet : Okzalik aside ve oksalatlara, bitkiler aleminde yaygın olarak rastlanmaktadır. Genellikle, bir metabolizma artığı olarak ortaya çıkan okzalik asidin dokularda birikimi, toksik etki yaratmaktadır. Bundan dolayı, oksalik asidi parçalayan enzimler yoğun ilgi çekmektedirler. Bu enzimlerden olan oksalat oksidaz'ın okzalik asidi parçalayarak CO_2 ve H_2O_2 ürettiği gösterilmiştir. H_2O_2 molekülünün bitki hücre farklılaşmasında, sinyal iletiminde ve hücre savunmasında rol alabileceği ifade edilmektedir. Koleoptil gelişimi sırasında ortaya çıkan germin proteinlerinin okzalat oksidaz enzimi ile yüksek oranda amino asit dizilim benzerliklerinin bulunduğu ve germin proteinlerinin de okzalat oksidaz aktivitesine sahip oldukları yapılan çalışmalarla gösterilmiştir.

Bitkilerde, koleoptil dokusunun oksinli ortamlarda bekletilmesi durumunda uzadığı bilinmektedir. Bu uzamanın mekanizması tam olarak anlaşılmamış olmakla beraber hücre duvarının bunda önemli bir rol oynadığı ileri sürülmektedir. Bir hücre duvarı proteini olan germin'in oksin hormonlarına duyarlı olması ve okzalat oksidaz aktivitesi göstermesi, onun bu mekanizmada bir fonksiyonu olabileceğini düşündürmektedir. Bu çalışma ile, oksinli ortamlarda bekletilen koleoptillerde germin-benzer okzalat oksidaz aktivitesinin oksinli ortamlarda bekletilmeyen koleoptillere göre daha yüksek olduğu gösterilmiştir.

Anahtar Sözcükler: Oksin, Germin-benzer okzalat oksidaz, Koleoptil gelişimi

Introduction

Oxalic acid, which is one of the strongest organic acids, and its salts, oxalates, are widely distributed within the cells and cell walls of plants. Oxalates were generally considered as an end-product of metabolism. However, the most recent studies have implied that they are involved in some metabolic processes (1). There are two possible enzymatic reactions for the degradation of oxalate in plants: (i) decarboxylation by oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2). (ii) oxidation by oxalate oxidase (oxalate: oxygen oxidoreductase EC 1.2.3.4). Oxalate oxidation has been found to occur in fungi, mosses and higher plants, but the biological significance of oxalate oxidation is not clear (2,3).

Recently, there has been growing evidence for the linkage of the developmental process to oxalate oxidase activity for oxalate degradation and the release of Ca^{++}

and H_2O_2 in plants (4). In particular the discovery that germin, a protein marker of early plant development, is an oxalate oxidase (5,6) suggested that oxalate oxidase, the enzymatic formation of H_2O_2 and Ca^{++} released from poorly soluble calcium oxalate might play an important role in metabolic regulation, particularly in cell-wall modification during germination and seeding development (7). As a product of germin-like oxalate oxidase activity, H_2O_2 might be involved in various metabolic processes; for instance at low concentrations it may act as a second messenger (8), whereas, at high concentrations it mediates the cross-linking of cell wall polymers, which restricts cell growth (9). Likewise, oxalate oxidase may affect the level of hydrogen peroxide, a compound necessary for cross-linking reactions (10), for example, lignification and conversion of ferulate to glucuronogalactoarabinoxylans, which bind selectively to oxalate oxidase and are allied with cereal wall modifications (11,12), Furthermore, H_2O_2 was localized at the site of papillae and hypersensitive response during the barley-powdery mildew interaction (13).

The discovery that germin gene expression is auxin responsive (14) immediately suggested the possible involvement of germin and its enzymatic activity in the action of auxin in plant doevelopment. The plant hormone auxin, typified by the naturally prevalent indoleacetic acid (IAA), is central to the control of plant growth and development. At the level of cellular physiology, auxin profoundly affects turgor, elongation, division and cell differentiation, the major driving and shaping forces in morphogenesis and oncogenesis. The molecular mechanisms of auxin action are still unknown, although it is now well established that auxin modulates membrane function and gene expression (15).However, a causal relationship between auxin-mediated alterations in gene expression or membrane function and a particular growth process has not yet been demonstrated.Germin-like oxalate oxidase as a cell wall glycoprotein (11) and the auxin responsive gene (14) seems to be the best candidate for analyzing auxin action during the plant cell growth (elongation). Auxin promotes the growth of coleoptiles by loosening epidermal cell walls, making them more susceptible to turgor-driven elongation (16). The changes in wall chemistry that underlie loosening are controversial. The determination and localization of germin-like oxalate oxidase activity in auxin-induced cell elongation which is the aim of the current study, might give us some clues about the possible mechanisms of auxin action.

Materials and Methods

Plant Material

Grains of spring wheat (*Triticum aestivum* L. var. Tonic) were obtained from Kenneth Wilson Grain, leeds, UK. Dry grains were surface sterilized by incubation in a 10% solution of domestic bleach (ca.1% free Cl_2) and five washes with sterile distilled water. Grains were germinated by incubation on two layers of water-soaked 3MM chromatography paper (Whatman) at 25ºC.

Determination of coleoptile elongation

Wheat embryos were germinated for 3 days. After 3 days, coleoptile segments (10 mm) were excised by cutting from a position 3 mm below the tip. These segments were preincubated in water at 25ºC for 1 hour in darkness, following which they (at least 20 coleoptile segments) were incubated in 50µl of 10µM IAA, 2% sucrose for auxin-induced coleoptile elongation at 25ºC for 12 hours in darkness. Control segments (at least 20 segments) were incubated in 2% sucrose solution lacking auxin and coleoptiles (at least 20) remaining on germinating embryos (3 days plus 12 hours) were used as a comparison with "normal" growth. The length of the coleeoptiles were measured at the start and end of the 12-hour incubation period.

Determination of Germin-like Oxalate Oxidase Activity

The oxalate oxidase activity of the above coleoptiles was determined by the method of Sugiura et al. (17). Plant tissues were homogenized in microcentrifige tubes with 0.1 ml extraction buffer (0.1M succinate buffer pH 3.5-2mM oxalic acid), and germin-like oxalate oxidase containing cell wall material recovered by centrifigation at 12,000xg for 2 minutes. The supernatants were used to determine the protein content by using the Bio-rad assay and the pellets were resuspended in 0.1 ml extraction buffer and incubated at 25ºC for 5-20 minutes, prior to the addition of 60µl developer (0.1M sodium phosphate buffer, pH 5.5, 40 units horseradish peroxidase, 0.25 mg 4-aminoantipyrine, 0.6 µl dimethylaniline). Following a further incubation for 10 minutes, the suspension was c larified by centrifugation (12,000 x g; 2 min) and the absorbance measured at 550nm. Control reactions lacked

oxalic acid in the homogenization buffer. Calibration was achieved by comparison with a set of standard H_2O_2 concentrations. The unit of enzyme activity was defined as the amount of enzyme which formed 1 umoles H_2O_2 per minute per mg of protein in assay. The results are the average of at least 20 repeats.

Histochemical Detection of Germin-like Oxalate Oxidase Activity

The enzymatic activity of germin-like oxalate oxidase was localized for IAA-treated and untreated coleoptiles as follows. Coleoptile segments were sectioned at-20ºC after freeze-embedding in OCT resin. Plant material was either fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), then washed with PBS, prior to embedding, or was embedded directly without prior fixation. Sections were mounted on poly-lysine coated microscope slides and incubated with 15 µl assay buffer (25 mM succinic acid, 3.5 mM EDTA, 2.5 mM oxalic acid, 0.6 mg/ml 4-chloro-1-naphthol, pH 4.0) at 25ºC until color development occurred (18). Control samples were incubated in assay buffer lacking oxalic acid. Prior fixation of sections was not found to be deleterious with respect to germin-like oxalate oxidase activity detection.

Electrophoretic Detection of Germin-like Oxalate Oxidase Activity

Wheat germin-like oxalate oxidase activity was analyzed in gels by using the Schagger gel method (19). Plant materials obtained from IAA-treated coleoptiles, sucrose-treated coleoptiles and intact coleoptiles left to grow on embryos were homogenized in water (1/1, w/v) and centrifuged at 12000 x g for 30 min. Protein content was determined by using the Bio-Rad assay to enable equal quantities of protein to be loaded in each lane. Following the electrophoresis, the gels were incubated in oxalate oxidase gel developer solution (20) until the colors appeared. The mini Bio-Rad gel apparatus was used.

Running Schagger Gel

Protein samples were mixed with 3 volumes of 4x loading buffer [SDS 2%,glycerol 6%, bromophenol blue 0.005%, tris (50 mM,pH:6.8) 25 mM] and loaded on the gel without reducing agent or prior heating. The gel was run at a constant voltage of 150 V for about 3 hours. After electrophoresis, the gel was immediately immersed in oxalate oxidase gel developer [Succinic acid/NaOH (oH:3.8) 40 mM,ethanol 60%, oxalic acid 2 mM, horse radish peroxidase 5 units/ml, 4-chloro-1-naphthol 50 mg/100 ml] and incubated at room temperature until activity staining appeared as a black band (generally overnight).

Results and Conclusion

The role of auxin in the elongation of coleoptile segments has been studies extensively over many years (21,22). The main controversy that developed during these studies was whether auxin functions in cell wall loosening through acidification of the cell wall caused by proton excretion by the cell (acid-growth; 16,23) or whether cell wall loosening mediated by auxin involves some other mechanisms such as synthesis of proteins required for the cell wall loosening process (24,25). Because germin-like oxalate oxidase was shown to be encoded by an auxin responsive gene (14), its possible involvement in IAA-iduced elongation of coleoptile segments was analyzed by measurement of the oxalate oxidase activity in enzyme assay buffers, staining of cryosections for localization of activity, and detection of oxalate oxidase activity in situ, in polyacrylamide gels.

Auxin-Induced Wheat Coleoptile Elongation and Germin-like Oxalate Oxidase

Coleoptile segments (10 mm in legth) were prepared from 3-day-old wheat seedlings and incubated in the presence or absence of IAA (10^{-6} M) as explained above. Both treated and untreated segments were found to increase in length over a 24-hour incubation period, but those incubated in the presence of IAA showed a 70% greater elongation than did the control segments (Figure 1). Following that, it was investigated how germin-like oxalate oxidase activity in auxin-induced coleoptile altered compared to normal growing coleoptiles. As shown in Figure 2, the auxin-treated coleoptile segments were found to have higher oxalate oxidase activity than the sucrose-treated control segments. Auxin application to coleoptiles raised the germin-like oxalate oxidase activity almost 2 times in these tissues comparing to untreated control coleoptiles (Figure 2). These results indicated that auxin induced the elongation of coleoptiles and increased the activity of germin-like oxalate oxidase in these tissues.

Determination of Auxin-Induced Germin-like Oxalate Oxidase Activity in Gels

Recently, methods for the detection and determination of oxalate oxidase have received

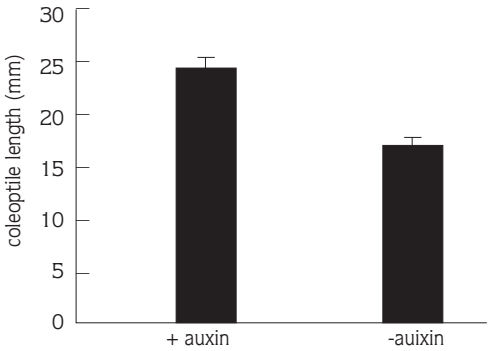

Figure 1. Determination of auxin effect on wheat coleoptile extension.

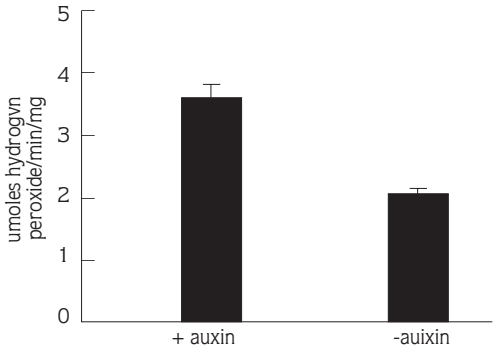

Figure 2. Determination of auxin effect on germin-like oxalate oxidase activity assay.

considerable attention, not only because this enzyme (and germin-like proteins, in general) has been implicated in a number of important phenomena in plant development and in plant defense (20,26), but principally because it forms the basis of a medical diagnostic procedure for the determination of oxalate in urine (a principal component of the formation of kidney stones) (27,28). When wheat germin was found to share a high homology with barley oxalate oxidase (5,6)-previously the only commercial source of oxalate oxidase for urinary oxalate detection kits, which rely on the production of the active oxypen specis, H_2O_2 from degradation of oxalate -an additional way of assaying for the presence of germin became available.

Since 1980, germins have been studied by immunological and hybridization methods (14). Identification of an enzymatic activity has enabled the development of rapid and sensitive colorimetric assays. The method developed by Zhang et al. (20) is probably the most efficient technique for determining oxalate oxidase activity, allowing detection both in situ in tissues, and in polyacrylamide gels. This represents an improvement on the methods of Sugiura et al. (17), Lane et al. (6) and Dumas et al. (18), which was achieved through including ethanol in the color developer solution. It has been claimed that this new method increases the sensitivity of detection up to 10-fold over the previous techniques. This method was therefore employed for the determination of the relationship between auxin and expression of germin-like oxalate oxidase activity in dissected coleoptile segments of wheat seedling. Two approaches were used. In the first, the oxalate oxidase activity in coleoptile segments was directly visualized following the resolution of homogenates in Schagger gels, taking advantege of the unique SDS-resistant properties of the germin oligomer. In the second, the activity was detected in situ in tissue sections, allowing the spatial distribution of the germin-like oxalate oxidase to be determined.

Protein extracts were prepared from auxin-treated coleoptile segment, sucrose-treated coleoptile segments and intact coleoptiles left to grow on embryos. Equal amounts of proteins were mixed with SDS-loading buffer lacking reducing agent and were loaded to Schagger gels without prior heat treatment. Following electrophoresis, germin-like oxalate oxidase was detected in the gels by incubating them in oxalate oxidase gel developer solution at room temperature overninght. The results revealed that Schagger gel systems were quite appropriate for resoling protein extracts and determining germin-like oxalate oxidase activity among protein extracts (Figure 3). When

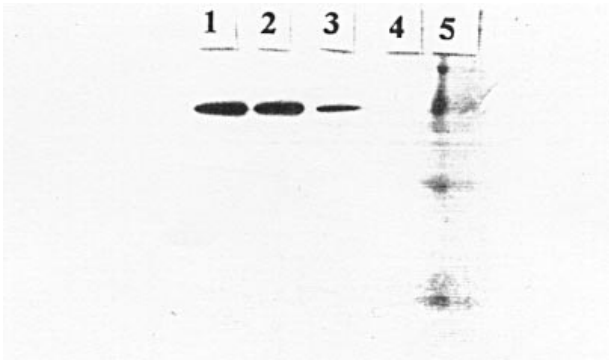

Figure 3. Determination of germin enzymatic activity in Shagger gel. Protein extracts were obtained from auxin-treated and untreated coleoptile segments as explained, resolved by electrophoresis and stained for oxalate oxidase activit. (1) Auxin-treated coleoptiles, (2) Sucrose-treated coleoptiles, (3) coleoptile segments without any treatments, (4) control, protein extracts from *Papaver somniferum* which does not express germin-like oxalate oxidase and (5) Markers.

equal amounts of protein extracts from auxin-treated, sucrose-treated and control coleoptiles were loaded in Schagger gel, both the auxin-treated and sucrose-treated coleoptile segments gave similar apparent levels of oxalate oxidase activity. However, the intact control coleoptiles showed a much lower level of oxalate oxidase activity, and the protein extracts from an irrelevant source "*Papaver somniferum L.*" did not give any activity band (Figure 3 lane 4). The marker used to determine the molecular weights of bands was resolved in gel developer

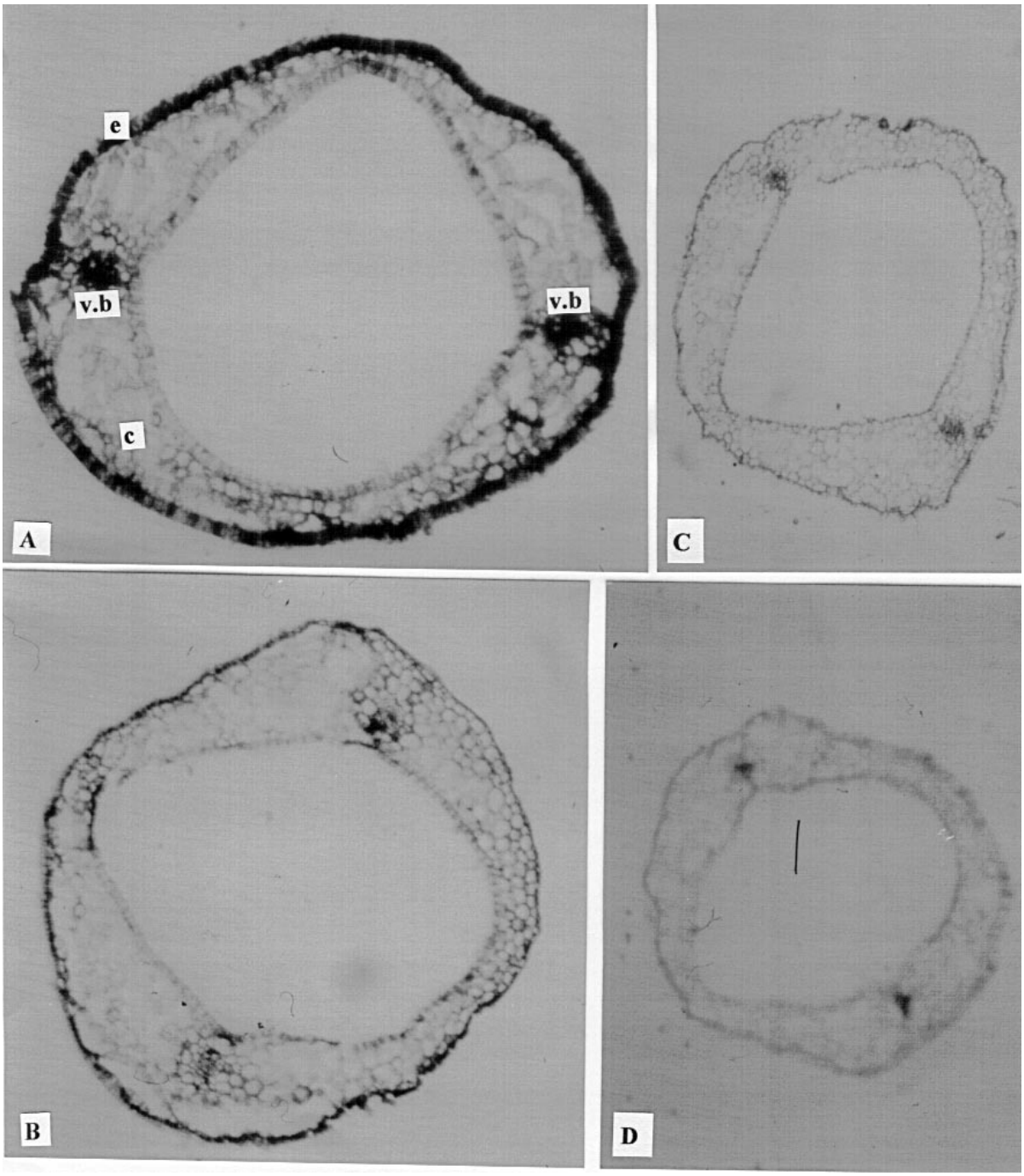

Figure 4. Localization of germin-like oxalate oxidase activity in auxin-treated coleoptiles. A: Auxin-treated coleoptile section, B: Sucrose-treated coleoptile section, C: Auxin-treated control section (oxalic acid omitted) and D: Sucrose-treated control section (oxalic acid omitted). e: epidermis, c: coleoptiles, v.b: vascular bundle

solution; therefore, the molecular weight of bands were obscure (Figure 3 lane 5). It was apparent that the Schagger gel system was satisfactory for searching for the germin-like oxalate oxidase activity in various tissues from different sources. However, it was not sensitive enough for observing the auxin effect on germin-like oxalate oxidase activity in coleoptiles.

Localization of Germin-like Oxalate Oxidase Activity during Auxin-Induced Coleoptile Elongation

The spatial distribution of germin-like oxalate oxidase was determined by preparing cryosections from coleoptile segment. The sections were stained for oxalate oxidase activity as described in Materials and Methods. Auxin-treated coleoptile sections (Figure 4A) showed an apparently higher level of oxalate oxidase activity than the sucrose-treated coleoptile sections (Figure 4B). In particular, the site of germin-like oxalete oxidase activity in coleoptiles was found to be the vascular bundles (Figure 4A). The outer epidermis cells clearly gave a stronger oxalate oxidase activity in auxin-treated coleoptiles than sucrose-treated coleoptiles (Figs. 4A and 4B). The epidermis is believed to be the growth limiting tissue in the coleoptile of the developing seedling so the association of germin accumulation and activity with this tissue is significant. No activity was detected in control coleoptile sections (Figure 4C and 4D). Germin might prepare this tissue for quick elongation upon auxin treatment by supplying necessary cell wall polymers cuch as hemicellulose groups or, alternatively, germin might act as a regulator in the epidermis to prevent further (or uncontrolled) tissue expansion upon auxin treatment by mediating cross-links between cell wall polymers throught its peroxide-generating enzyme activity.

In the current study, germin-like oxalate oxidase activity was found to increase during the auxin-induced coleoptile elongation. The role of germin-like oxalate oxidase in auxin- induced cell elongation is not clear. However, auxin might have a dual action during cell elongation. The first action is the induction of elongation by loosening cell wall polymers and the second action is the termination of elongation by inducing germin-like oxalate oxidase activity. It is known that H_2O_2 as a product of germin-like oxalate oxidase, restricts cell elongation by mediating the cross-linking of cell wall polymers.

References

1. Caliskan, M., The metablism of Oxalic acid, Tr.J.Zoology 24:103-106 (2000)
2. Hodgkinson, A., Oxalic Acid in Biology and Medicine, Academic Press, London (1977).
3. Franceschi, V.R., Horner, J_R.H.T., Calcium oxalate crystals in plant, The Botanical Review 46: 361-427 (1980).
4. Lane, B.G., Cuming, A.C., Fregeau, J., Carpita, N.C., Hurkman, W.J., Bernier, F., Dratewka-Kos, E., Kenndy, T.D., Germin isoforms are discrete temporal markers of wheat development: pseudogermin is uniquely thermostable water-soluble oligomeric protein in ungerminated embryos and like germin in germinated embryos, it is incorporated into cell walls, Eur. J.Biol. Chem. 209:961-969 (1992).
5. Dumas, B., Sailland, A., Cheviet, J-P., Freyssinet, G., Pallett, K., Identification of barley oxalate oxidase as a germin-like protein, C.R., Acad. Sci.Paris, Sciences de la vie/Life sciences 316:793-798 (1993).
6. Lane, B.G., Dunwell, J.M., Ray, J.A., Schmitt, M.R., Cuming, A.C., Germin, a protein marker of early plant development, is an oxalate oxidase, J. Biol. Chem 268: 12239-12242 (1993).
7. Lane, B.G., Oxalate, germin, and the extracellular matrix higher plant, FASEB J. 8:294-301 (1994).
8. Luttrel, B.M., The biological relevance of the binding of calcium ions by inositol phosphates, J.Biol. Chem. 268:1521-1524 (1993).
9. Showalter, A.M., Structure and function of plant cell wall proteins, The Plant Cell 5: 9-23 (1993).
10. Hohl, M., Greiner, H., Schopfer, P., The cryptic-growth response of maize coleoptiles and its relationship to H_2O_2 dependent cell wall stiffening, Physiol. Plantarum 94:491-498 (1995).
11. Jaikaran, A.S.I., Kenndy, T.D., Dratewka-Kos, E., Lane B.G., Covalently bonded and adventitious glycans in germin, J.Biol. Chem. 265:12503-12512 (1990).
12. Carpita, N.C., Gibeaut, D.M., Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth, The Plant Journal 3:1-30 (1993).
13. Thordal-Christensen, H., Zhang, Z., Wei, Y., Collinge, D.B., Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction, The Plant Journal 11:1187-1194 (1997).
14. Calişkan, M., Cuming, A.C., Spatial specificity of H_2O_2-generating oxalate oxidase gene expression during wheat embryo development. The Plant Journal 15: 165-171 (1998).

15. Napier, R.M., Venis M.A., Auxin action and auxin-binding proteins, New Phytol. 129:167-201 (1995).

16. Kutschera, U., The current status of the acid-growth hypothesis, New phytol. 126: 549-569 (1994).

17. Sugiura, M., Yamamura, H., Hirano, K., Sasaki, M., Morikawa, M., Tsuboi, M., Purification and properties of oxalate oxidase from barley seedlings, Chem. Pharm. Bull. 27: 2003-2007 (1979).

18. Dumas, B., Freyssinet, G., Pallett, K.E., Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings, Plant Physiol. 107:1091-1096 (1995).

19. Schagger, H., von Jagow, G., Tricine, sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 Kda., Anal. Biochem. 166:368-379 (1987).

20. Zhang, Z., Yang, J., Colling, D.B., Thordal-Christensen, H., Ethanol increases sensitivity of oxalate oxidase assays and facilitates direct activity staining in SDS gels. Plant Mol. Biol. Reporter 14:266-272 (1995).

21. Kutschera, U., Schopfer, P., Evidence against the acid-growth theory of auxin action, Planta 163:483-493 (1985).

22. Rayle, D.L., Cleland, R., The acid growth theory of auxin-induced cell elongation is alive and well, Plant Physiol. 99:1271-1274 (1992).

23. Rayle, D.L., Cleland, R., Evidence that auxin-induced growth of soybean hypocotyls involves proton excretion, Plant Physiol. 66:433-437 (1980).

24. Edelmann, H.G., Schopfer, P., Role of protein and RNA synthesis in the initiation of auxin-mediated growth in coleoptiles of *Zea mays* L., Planta 179:486-494 (1989).

25. Schindler, T., Bergfeld, R., Hohl, M., Schopfer, P., Inhibition of Golgi-apparatus function by brefeldin A in maize coleoptiles and its consequences on auxin-mediated growth, cell-wall extensibility and secretion of cell-wall proteins, Planta 192:404-413 (1994).

26. Hurkman, W.J., Tanaka, C.K., Germin gene expression is induced in wheat leaves by powdery mildew infection, Plant Physiol. 111:735-739 (1996).

27. Laker, M.F., Hofmann, A.F., Meeuse, B.J., Spectrophotometric determination of urinary oxalate with oxalate prepared from moss, Clin. Chem. 26: 827-830 (1980).

28. Obzansky, D.M., Richadson, K.E., Quantification of urinary oxalate with oxalate oxidase from beet stems. Clin. Chem 29:1815-1819 (1983).