Effects of Exogenous Auxins on Expression of Lipoxygenases in Cultured Soybean Embryos

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ABSTRACT

The expression of lipoxygenases (LOXs) is known to be developmentally regulated in soybeans (Glycine max. [L.] Merr.). Hormones have been firmly established as being involved in the growth and developmental processes of a number of plant species. Correlation between the expression of LOXs and the development and germination of soybean embryos suggests that plant hormones may affect the expression of LOXs. The present studies were conducted to investigate the effects of exogenous auxins on the expression of LOX isoforms and LOX activities in cultured cotyledon tissues of immature soybean seeds. The results revealed that at least one of the more acidic nonembryonic LOX isoforms was induced by either α-naphthylacetic acid or indoleacetic acid but not by 2,4-dichlorophenoxyacetic acid after 4 days¹ exposure. Levels of LOX-1, -2, and -3 proteins and activities were significantly decreased by 2,4-dichlorophenoxyacetic acid 10 days after planting. S1 analysis showed that embryo LOX messenger RNAs were detectable in the tissues treated with each of the auxins. The reduced levels of the embryo LOX proteins may, therefore, be regulated at the levels of translation, posttranslational modification, or degradation. The more acidic isoforms induced by α-naphthylacetic acid showed enzymatic activity and shared the same molecular mass and isoelectric point values as the germination-associated LOX isoforms found in hypocotyls and radicles, suggesting that these LOXs are involved in germination competency of soybean embryos.

LOXs² (EC 1.13.11.12) are commercially important enzymes that catalyze the peroxidation of molecules containing cis,cis-1,4-pentadiene moieties (13) and appear to be ubiquitous among eukaryotic organisms. In animals, LOXs catalyze the oxygenation of arachidonic acid released from phospholipids. The LOX-derived metabolites participate as signals in several biochemical and physiological processes (32). In plants, however, no clearly defined physiological role has been demonstrated for LOXs, although several possibilities have been proposed recently (15, 16).

At least seven isoforms of LOX have been found to date. Three isoforms have been isolated from soybean seed and designated LOX-1, -2, and -3 (2). Four additional isoforms, designated LOX-A, -B, -C, and -D were found in germinated seedlings (23, 26). At least three LOX isoforms have also been detected in soybean leaves using leaf LOX antibodies, which appear to be different from LOX-1, -2, and -3 (12). All of the isoforms identified have a similar molecular mass of approximately 95 kD but different pi values. It is well documented that the expression of LOXs is tightly regulated during in planta soybean zygotic embryo development and seed germination in soybean (1, 2, 16, 22) and seed development in Pisum (5), suggesting that LOXs have a role(s) in these processes. Hildebrand et al. (17) reported that expression of LOX-1, -2, and -3 is undetectable during the early stage of zygotic embryo development but shows a dramatic increase later in embryo development. This is supported by the finding of a correlation between expression of LOXs and induction and development of somatic embryos in soybeans (21).

It has been reported that the levels of plant hormones change greatly during the development of zygotic embryos in a number of species. Several investigators have reported changes in auxins during plant embryo development. Studies with developing soybean and Phaseolus vulgaris embryos (4, 14) indicate that free IAA peaks at the stage of maximal embryo cell division and pod expansion. Later in embryo development, most of the IAA becomes conjugated into amide linkages such as glutamate (4, 7). Free IAA is reduced at about the time of the large increase in embryo-specific LOXs (17). However, during germination free IAA levels increase again (4).

Successful somatic embryogenesis from immature zygotic embryo cotyledons of soybean has been accomplished via exogenous auxin application at high concentrations (e.g. 2,4-D at 40 mg/L) (8, 20), suggesting that auxin is a key regulator of embryo induction. Auxins may act either by turning on the expression of certain genes or by being involved in the modification of key gene products. The fact that the expression of embryo LOX is developmentally regulated indicates that auxin may influence the expression of LOXs. The objective of the present study was to investigate the effects of exogenous auxins on the expression and enzymatic activity of LOXs in cultured soybean embryo tissues.

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³ Abbreviations: LOX, lipoxygenase; NAA, α-naphthylacetic acid; TBS, Tris-HCl 0.05 m and NaCl 0.2 m; RIPA, Tris-HCl 0.05 m, NaCl 0.15 m, EDTA 0.004 m, SDS 0.1%, Triton X-100 1%, and sodium deoxycholate 1%; pl, isoelectric point; IEF, isoelectric focusing; bp, base pair; H/R, hypocotyl/radicle; MSO, Murashige and Skoog salts, B₆ vitamins, 2% (w/v) sucrose, pH 5.8, and 0.2% (w/v) Gelrite.

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MATERIALS AND METHODS

Plant Materials and Culture Medium

Pods of soybean Glycine max (L.) Merr. cv ‘Century’ were collected from plants grown in the greenhouse and surface sterilized with 70% 2-propanol for 2 min, followed by 1% sodium hypochlorite (20% Clorox bleach) for 10 min, and rinsed (four times) in sterile water. Immature seeds, 3 to 5 mm in length, were aseptically removed from pods, and the end containing the embryonic axis was cut off and discarded. The two cotyledons were pushed from the seed coat and separated; one was placed abaxial side down on MSO medium (Murashige and Skoog salts, B1 vitamins, 2% [w/v] sucrose [pH 5.8], and 0.2% [w/v] Gelrite) and the other on NAA 10 medium (MSO plus 10 mg/L NAA) or 2,4-D 40 medium (MSO plus 40 mg/L 2,4-D). Cultures were incubated at 25°C with a 23-h photoperiod (approximately 8 \( \mu \)E/m\(^2\)/s) which was used for somatic embryogenesis. Explant tissues and induced somatic embryos were harvested at 2, 4, 6, 8, 10, 15, 20, and 30 d after being inoculated on the different media, frozen in liquid nitrogen, and stored at −70°C for protein analysis. To investigate the effects of different auxins, the cotyledons from seeds 8 to 9 mm in length, with the axis end of the cotyledons and embryonic axes removed, were placed on MSO medium containing various auxins at the levels of NAA 10, 25, and 50 mg/L; 2−4-D 0.4, 4, and 40 mg/L, and IAA 10, 40, and 100 mg/L. MSO and 0 time incubation were used as controls. Factorial randomized complete blocks with 3 replications were used. Cultures for each replication were set up on different days with days, therefore, representing blocks. Explants were harvested from each treatment at 5 and 10 d for RNA isolation and frozen in liquid nitrogen for protein analysis. Embryogenic suspension cultures (8) were utilized for the proliferation of soybean cv ‘Fayette’ somatic embryos. MSO medium either with or without 0.05 mg/L IAA and 0.05 mg/L 6-benzylaminopurine (0.05 IAA + BA or MSO) was used for growth of the somatic embryos. Soybean seeds were germinated according to the method of Park and Polacco (23). Cotyledons and hypocotyls were harvested from 1- to 7-d-old seedlings and stored at −70°C as tissue sources.

Protein Extraction

All tissues, stored at −70°C, were ground in chilled mortars with 5 volumes of deionized water per fresh weight. The extracts were centrifuged at 13,600g for 15 min at 0 to 4°C. Supernatants were transferred into fresh chilled Eppendorf tubes and subjected to two additional 15-min centrifugations as above. The resulting supernatants were used for protein assays. Proteins of the tissue extracts were quantified by a modified Lowry method (3), using bovine serum albumin as the standard.

IEF and SDS-PAGE

Native IEF was performed as described by Funk et al. (10), using a 5% polyacrylamide gel and an LKB flatbed system. Electrophoresis was carried out at 6 W constant power, 20 mA, and 34,000 volt hours. SDS-PAGE was performed according to the method of Laemmli (18) using 10% running and 4% stacking gels. The gels were either stained with Coomassie brilliant blue G or analyzed by Western blotting.

Western Blotting

SDS and IEF gels were Western immunoblotted as described by Wang and Hildebrand (31) with minor modifications. Gels were electroblotted to nitrocellulose at 50 mA overnight. After blotting, the nitrocellulose filters were incubated in 20% (v/v) dry milk in TBS at room temperature for 4 h, washed with TBS containing 0.25% (w/v) gelatin and 0.5% (w/v) Tween 20 for 10 min, followed by RIPA and TBS for 10 min each, and then incubated in a mixture of LOX-1, -2, and -3 rabbit antibodies at dilutions of 1:1,000, 3:10,000, and 3:10,000, respectively, overnight. After washing as above, the nitrocellulose was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG in 10% (w/v) blocking milk for 4 h, followed by rinsing as above, and then stained with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitro-blue tetrazolium chloride staining solution. The limits of detection using these antibodies and techniques are about 0.4, 2, and 1 pmol for LOX-1, -2, and -3, respectively (17).

In-Gel Activity Staining

IEF gels were stained for LOX activity according to the procedure of Park and Polacco, (23) with minor modifications. Just before staining, 20 mg o-dianisidine (3,3'-dime-thoxybenzidine; Fast Blue B Base) in 14 mL 100% ethanol and 100 mg linoleic acid sodium salt in 2 mL 50% (w/w) ethanol were mixed in 50 mL 0.2 M phosphate buffer (pH 7.0), subsequently diluted to 100 mL using deionized water, and then 100 μL of 1 M KCN in deionized water was added. The IEF gel was incubated with this mixture in the dark overnight.

Activity Measurement

LOX activity was determined by spectrophotometric measurement of the formation of conjugated dienes at 235 nm (2). The combined activity of LOX-2 and -3 (type II) was determined at pH 6.8, and LOX-1 activity (type I) at pH 9.0, using sodium linoleic acid as the substrate. Both substrate solutions were prepared immediately before assay and kept in a light-proof container on ice during use. All substrate solutions were adjusted to an absorbance of 0.45 at 235 against an appropriate buffer blank, using partially oxidized substrate. This was done to standardize hydroperoxylinoleic acid levels, which would vary otherwise due to autooxidation. Hydroperoxyoctadecadienoic acid sometimes described as the LOX activator (9) is commonly used to activate LOXs.

Extraction of RNA and S1 Nuclease Analysis

Total plant RNA was isolated by acidified guanidinium thiocyanate extraction (24) from 8- to 9-mm zygotic embryo cotyledon explant tissues treated with different growth regulators at various levels. An additional chloroform extraction was used in our experiments. Growth regulator treatments of embryo cotyledons were the same as those used for protein
analysis. Wheat germ tRNA (Sigma) was used as a negative control. Before use, plant RNA was fractionated on a 2% agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 0.002 M EDTA) to verify that the RNA was largely intact. This was indicated by the presence of multiple bands representing abundant RNA species.

LOX isozyme-specific probes were prepared from the 3' region of the cDNAs which showed high divergence. The LOX-1 clone used in these studies consisted of the 174-bp EcoRI to SstI fragment from pMX85 (28) ligated to M13mp18 (33) that was also digested with EcoRI and SstI. The LOX-1 DNA corresponds to nucleotides 2578 to 2751 on the LOX-1 cDNA sequence of Shibata et al. (28).

The LOX-2 clone contained the 107-bp HindII to XhoII fragment from pLX-65 (29). M13mp19 (33) was digested with SphI, and the second DNA strand was synthesized with the Klenow fragment of DNA polymerase I. M13mp19 was also digested with BamHI (an isoschizomer of XhoII). The digested LOX-2 fragment was ligated to the digested M13mp19. The LOX-2 DNA in this clone corresponds to nucleotides 2646 to 2752 on the LOX-2 cDNA sequence of Shibata et al. (27). The clone for LOX-3 used for S1 nuclease analysis consisted of the 221-bp EcoRI to HindII fragment from pLX-10 (29) ligated to M13mp18 (33) that was digested with EcoRI and AccI. The LOX-3 DNA corresponds to nucleotides 4929 to 5149 on the LOX-3 sequence of Yenofsky et al. (34).

Single-stranded DNA was prepared from M13, and probes were uniformly labeled using primer extension. The probes for LOX-1 and LOX-3 were cut with EcoRI, the probe for LOX-2 was cut with HindIII, and the fragments containing LOX sequences were eluted from gels. [$^{32}$P]dCTP-labeled probe containing 60,000 Cerenkov cpm was hybridized to 5 μg total plant RNA (or tRNA). DNA size markers were prepared by end labeling 1-kilobase ladder DNA (BRL) with T4 DNA polymerase according to manufacturer's instructions. Other details of hybridization, S1 nuclease analysis, and gel conditions were described previously (25).

RESULTS

Effects of Exogenous 2,4-D and NAA on LOX Expression in 3- to 5-mm Zygotic Embryo Cotyledon Explants

Either very low or no detectable levels of LOX-1, -2, and -3 were found in the cotyledons of immature seeds 3 to 5 mm in length (Fig. 1A, lane 2; Fig. 1B, lane 2). When the cotyledon tissues were cultured on either MSO or NAA 10 medium, all of the isozymes increased dramatically in the tissues by 4 d after explanting (Fig. 1A, lanes 6 and 7; Fig. 1B, lanes 5 and 6).

![Figure 1](https://www.plantphysiol.org)
This is not unexpected because excised cotyledons can undergo maturation by continuing dry matter accumulation in an in vitro culture system (6). It is, however, surprising that several more acidic polypeptides cross-reacting with seed LOX antibodies were found in the tissues cultured on NAA (Fig. 1A, lanes 7, 9, and 11), but they were not detected in the tissues cultured on either MSO (Fig. 1A, lanes 6, 8, and 10) or 2,4-D medium (Fig. 1A, lane 3). Generally, these acidic isozymes shared the same molecular mass(es) and pI value(s) as germination-associated LOXs (Fig. 1A, lane 1; Fig. 1B, lane 1; and Fig. 1C, lanes 6 and 7). However, at least one isozyme expressed in the H/R tissues 7 d after imbibition was not detectable in the tissues treated with 10 mg/L NAA (Fig. 1A, lane 1). SDS-PAGE analysis showed that the dominant isozymes expressed in the tissues cultured on NAA had a molecular mass of about 95 kD, but some polypeptides with lower molecular masses were also detected (Fig. 1B, lanes 6 and 8). Some of them shared the same molecular mass as the polypeptides detected in the radicle tissues 7 d after imbibition. SDS-PAGE of the acidic bands and LOX-1 isolated from IEF gels confirmed that the acidic isozymes have a molecular mass of about 95 kD (Fig. 1B, lanes 9 and 10). In-gel activity staining showed that the induced acidic isozymes could catalyze the conversion of linoleic acid to hydroperoxide (Fig. 1C). None of the LOX isozymes were detectable in the tissues treated with 4 mg/L 2,4-D after 6 d (Fig. 1A, lane 3).

Effects of Exogenous Auxins on Expression of LOXs in 8- to 9-mm Zygotic Embryo Cotyledon Explants

LOX-1, -2, and -3 were abundant in the cotyledon tissues of soybean seeds 8 to 9 mm in length and in somatic embryos (Fig. 2A, lanes 1 and 12). Therefore, if exogenous auxins have effects on the expression of LOXs in soybean tissue cultures as described above, then the levels of LOXs in 8- to 9-mm zygotic embryo cotyledons and somatic embryos should change after the tissues are cultured on media containing various auxins at different levels. IEF and SDS gel Western blots showed that, after 10 d culture on media containing either 40 or 4 mg/L 2,4-D, no LOX isozymes were detectable in the tissues (Fig. 2A, A and B, lanes 3 and 4). The lowest level of 2,4-D (0.4 mg/L), NAA or IAA did not eliminate the expression of LOXs (Fig. 2A, A and B). Compared with MSO, 10 mg/L NAA resulted in an increase in the protein levels of embryo LOXs. High levels of NAA (50 mg/L) and IAA (100 mg/L) decreased LOX levels in the cotyledon tissues (Fig. 2B, lanes 8 and 9). However, expression of acidic LOX bands was seen on NAA and IAA (Fig. 2A, lanes 6–11) as was the case with 3- to 5-mm cotyledons on NAA (Fig. 1A). To further investigate the effects of the auxins on LOX expression, S1 nuclease protection analysis was performed. The results presented in Figure 3 show that transcripts for LOX-1 (Fig. 3A), -2 (Fig. 3B), and -3 (Fig. 3C) are expressed in all of the tissues tested, including the tissues cultured on medium containing 100 mg/L IAA, 10, 25, or 50 mg/L NAA, or 0.4, 4, or 40 mg/L 2,4-D. Similar results were obtained in two additional replicate experiments as well as from tissues cultured on the same media for 20 d (data not shown). The nondetectable levels of LOX isozymes were not caused by activation of general proteolytic activity, because most of the extracted proteins were unchanged by auxin treatment (Fig. 2C). However, SDS-polyacrylamide gels stained with Coomassie blue (Fig. 2C) showed that, compared with MSO (lane 1), 2,4-D at...
in the tissues cultured on the medium containing 40 or 4 mg/L 2,4-D for the same period (Fig. 4, lanes 1 and 2). It was very interesting that two of the acidic polypeptides induced in 3- to 5-mm cotyledon explants were still found in 8- to 9-mm cotyledon tissues cultured on either NAA or IAA medium (Fig. 2A, lanes 6–11; Fig. 4, lanes 4–9) but were not found in the tissues cultured on either 2,4-D (Fig. 2A, lanes 3–5; Fig. 4, lanes 1–3) or MSO (Fig. 2A, lane 2; Fig. 4, lane 10) medium. The relative levels of these isozymes were much lower than those found in 3- to 5-mm cotyledon tissues with the same treatment (Fig. 1A).

Effects of Exogenous Auxins on LOX Activity

Five days after treatment, all of the auxins resulted in a decrease of type II LOX activity (LOX-2 and -3 at pH 6.8) (Fig. 5A). The greatest decrease of LOX activity was caused by 2,4-D at 40 mg/L. Compared with the activity measured in the initial explant tissues, type I activity (LOX-1 at pH 6.8) was increased on 40 mg/L IAA and MSO media. Type I activity was only decreased by 40 mg/L 2,4-D, whereas all treatments reduced type II activity. At day 10, both type I and type II activities were significantly decreased in the tissues treated with either 2,4-D or high concentrations of NAA or IAA, with nondetectable LOX activity in the tissues treated with 40 mg/L or 4 mg/L 2,4-D (Fig. 5B). Auxins resulted in a larger decrease of type I activity than that of type II activity in most cases 10 d after treatment, although higher concen-

40 mg/L (lane 2) and 4 mg/L (lane 3) resulted in altered profiles of some of the extracted proteins. Several polypeptides with molecular masses of about 34, 32, and 27 kD were induced by 40 mg/L 2,4-D (Fig. 2C). These polypeptides could have resulted from specific proteolytic degradation of storage proteins or from degraded LOXs.

Although the levels of LOX-2 and -3 clearly decreased in the tissues treated with 4 or 40 mg/L 2,4-D after 5 d (Fig. 4, lanes 1 and 2), LOX-1 was still present at a relatively high level in the tissues. One of the acidic bands, which was detected in 8- to 9-mm zygotic embryo cotyledons and sometimes in somatic embryos (Fig. 2A, lanes 1 and 12), disappeared in the tissues cultured on MSO, NAA, and IAA media 10 d after explanting (Fig. 2A, lanes 2–11) but was still detectable in the tissues cultured on the medium containing 0.4 mg/L 2,4-D, NAA, IAA, or no growth regulators for 5 d (Fig. 4, lanes 3–10). This isozyme, however, was not detected

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Figure 3. S1 nuclease protection studies of LOX gene expression. tRNA, wheat germ tRNA; control, RNA from 8–9 mm zygotic embryo cotyledons before culture. The remaining RNA samples are from 10-d cultures without added hormones (MSO) or with added IAA, 2,4-D, or NAA. A, probe = LOX-1; B; probe = LOX-2; C, probe = LOX-3. Size markers, nucleotide base pairs of DNA. Arrowheads indicate where LOX-1, LOX-2, or LOX-3 appear.

Figure 4. IEF gel Western blot analysis of the effect of exogenous auxins on expression of LOXs in 8- to 9-mm zygotic embryo cotyledon explant tissues cultured for 5 d on 2,4-D at 40 (lane 1), 4 (lane 2), and 0.4 mg/L (lane 3); NAA at 10 (lane 4), 25 (lane 5), and 50 mg/L (lane 6); IAA at 100 (lane 7), 40 (lane 8), and 10 mg/L (lane 9); and MSO (lane 10) (50 µg protein loaded per lane). Arrowheads indicate where acidic LOXs appear.
trations of 2,4-D greatly reduced both type I and II activities. Compared with MSO, low concentrations of NAA (10 mg/L) and IAA (10, 40 mg/L) did not significantly affect type II activity of LOXs as did 2,4-D or higher concentrations of NAA or IAA. In-gel activity staining showed that the activity of LOX-2 was much lower than that of LOX-3 in cultured 3- to 5-mm cotyledon tissues (Fig. 1C, lanes 1–3). Taken together, the decrease in LOX activities caused by 2,4-D or high concentrations of NAA or IAA is apparently due to a reduction in the levels of the LOX proteins (Figs. 2A, 2B, and 4).

DISCUSSION

Using a mixture of LOX-1, -2, and -3 rabbit antibodies, we have demonstrated that exogenous auxins have significant effects on levels of LOX isozymes in cultured soybean cotyledon tissues. The fact that several more acidic LOX isozymes are found in the tissues cultured on NAA indicates that NAA directly or indirectly induced the expression of these acidic LOXs. However, we currently do not know how NAA affects the expression of the LOXs, but the acidic LOXs induced by NAA are probably due to the expression of LOX gene(s) different from LOX-1, -2, and -3. Germination-associated LOXs with more acidic pI values have been reported, and their cDNA clones have been isolated (22, 26). Amino acid sequences of the predicted protein have been shown to be different from all LOXs sequenced so far including the embryo-specific LOXs (LOX 1–3). Whether the more acidic LOXs induced by NAA are identical with the germination-associated LOXs needs to be assessed.

It has been demonstrated that somatic embryos induced by NAA usually exhibit normal morphology, although the frequency of embryogenesis and the mean number of embryos per responding explant is lower on NAA medium than on 2,4-D medium (19). Somatic embryos with more normal morphologies are easier to convert into plants via somatic embryo germination. This implies that NAA may, via induction of some germination-specific proteins including the acidic LOXs, increase the germination frequency and/or that these acidic isozymes of LOX may play a role(s) in the germination of soybean embryos. If this is true, then the most difficult obstacle in plant regeneration via somatic embryogenesis in soybean—conversion of somatic embryos to plants—could be improved by systems that stimulate expression of these genes. In addition, some light could be shed on the mechanism by which plants regulate growth and developmental processes by understanding the functional roles of such germination-specific proteins.

Except for the dominant LOXs resolved by the SDS-PAGE Western blot, several polypeptides were found that cross-reacted with the mixture of LOX-1, -2, and -3 rabbit antibodies in the germinating seedling tissues and the tissues cultured on NAA (Fig. 1B) but not in the tissue cultured on MSO. Whether these species are the products of general proteolytic activity, and/or related to processes such as germination, is still unknown. Further studies will need to be conducted to characterize the acidic LOXs and the polypeptides with low molecular masses induced by NAA.

Compared with those in 8- to 9-mm cotyledon explant tissues, the relative levels of the more acidic LOX isozymes were much higher in 3- to 5-mm cotyledon explant tissues (Figs. 1A and 2A). If the acidic LOXs are the result of the expression of a new gene(s), then the evidence suggests that the expression of the more acidic isozymes may be under control of some internal factors that can only be induced (e.g. by NAA) from a certain type of cell or from the cells at certain
stages. What the factors are, how they can be induced, and why they can be induced only in certain cells remain to be determined. Because IAA also resulted in the expression of the more acidic isozymes, we suspect that IAA may be one of the endogenous intermediates in the induction of the expression of the acidic LOXs. These 3- to 5-mm soybean zygotic embryos are at the cotyledon stage of embryo development which is at the end of cell division and differentiation of embryo axis and cotyledons (11). Free IAA is high at this stage, decreases at later stages of development, and apparently increases again during germination (4, 7, 14).

Our data show that 2,4-D results in different effects on the expression of LOXs, in contrast to NAA and IAA. First, there are no detectable acidic isozymes found in the tissues treated with 2,4-D. Second, 2,4-D at 40 or even 4 mg/L eliminated detectable expression of LOX. This was not found with NAA and IAA at the concentrations used. As mentioned in “Results,” the significant decrease in LOX activities in the tissue treated with high levels of auxins is partially due to decreased levels of LOX proteins. However, we do not know which other factors are affecting LOX activities in the tissues where the levels of LOX proteins were not significantly decreased (Fig. 1A, lanes 6–11; Fig. 2A, lanes 5–11), but LOX activities were lower (Fig. 1C, lanes 1–5; Fig. 5B). The fact that type I activity (LOX-1) is greatly increased 5 d after expelling on 40 mg/L IAA and MSO media (Fig. 5A), compared with that in the initial explant tissues, indicates that, upon hydration or activation by some components in the medium or dilution of endogenous regulatory compounds, LOX activity can be altered. Wang et al. (30) reported that culture of soybean zygotic embryos can affect LOX activity.

Although we have clearly demonstrated that higher levels of 2,4-D can decrease LOX proteins to undetectable levels, how 2,4-D acts in this process(es) remains to be determined. However, detection of the mRNAs for LOX-1, -2, and -3 in the tissues cultured at higher levels of 2,4-D indicates that the expression of LOX isozymes may be reduced by lower translation, posttranslational modification, or enhanced degradation. Further studies such as pulse-chase labeling with $^{35}$S are needed to help resolve this issue.

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