Plant Physiol. (1992) 100, 231–237
0032-0889/92/100/0231/07/$01.00/0

Received for publication January 28, 1992
Accepted April 4, 1992

Regulation of Ascorbate Oxidase Expression in Pumpkin by Auxin and Copper[1]

Muneharu Esaka*, Kouichi Fujisawa, Miwa Goto, and Yasutomo Kisu

Laboratory of Enzyme Chemistry, Faculty of Applied Biological Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima, 724 Japan

ABSTRACT

Ascorbate oxidase expression in pumpkin (*Cucurbita* spp.) tissues was studied. Specific ascorbate oxidase activities in pumpkin leaf and stem tissues were about 2 and 1.5 times that in the fruit tissues, respectively. In seeds, little ascorbate oxidase activity was detected. Northern blot analyses showed an abundant ascorbate oxidase mRNA in leaf and stem tissues. Fruit tissues had lower levels of ascorbate oxidase mRNA than leaf and stem tissues. Ascorbate oxidase mRNA was not detected in seeds. Specific ascorbate oxidase activity gradually increased during early seedling growth of pumpkin seeds. The increase was accompanied by an increase in ascorbate oxidase mRNA. When ascorbate oxidase activity in developing pumpkin fruits was investigated, the activities in immature fruits that are rapidly growing at 0, 2, 4, and 7 d after anthesis were much higher than those in mature fruits at 14 and 30 d after anthesis. The specific activity and mRNA of ascorbate oxidase markedly increased after inoculation of pumpkin fruit tissues into Murashige and Skoog's culture medium in the presence of an auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) but not in the absence of 2,4-D. In the presence of 10 mg/L of 2,4-D, ascorbate oxidase mRNA was the most abundant. Thus, ascorbate oxidase is induced by 2,4-D. These results indicate that ascorbate oxidase is involved in cell growth. In pumpkin callus, ascorbate oxidase activity could be markedly increased by adding copper. Furthermore, immunological blotting showed that the amount of ascorbate oxidase protein was also increased by adding copper. However, northern blot analyses showed that ascorbate oxidase mRNA was not increased by adding copper. We suggest that copper may control ascorbate oxidase expression at translation or at a site after translation.

Ascorbate oxidase (EC 1.10.3.3) is a copper-containing blue enzyme that has been studied in plant tissues such as pumpkin (17, 29), cucumber (24), and orange (31). The enzyme catalizes the oxidation of ascorbic acid to dehydroascorbic acid. Recently, the enzyme has been used for clinical and food analyses of L-ascorbic acid (8, 20).

The definitive biological function of ascorbate oxidase is not clear, although it has been reported that the enzyme may participate in a redox system involving ascorbic acids (32). Furthermore, the subcellular localization of ascorbate oxidase is uncertain. The enzyme may be localized in the cell wall (13, 22, 25), although there have been some reports that the enzyme is localized in the cytoplasm (33), microsomes (33), or vacuole (31). Recently, Lin and Varner (18) studied the expression of ascorbate oxidase in zucchini and reported that the enzyme may be involved in reorganization of the cell wall.

We have studied the ascorbate oxidase in cultured pumpkin (*Cucurbita* spp.) cells and in a previous paper reported that ascorbate oxidase activity rapidly increased during growth of pumpkin callus (6). Furthermore, ascorbate oxidase was hown to be secreted into the culture medium in the cell suspension cultures (4), supporting the idea of cell wall localization of ascorbate oxidase. In addition, calcium ions markedly stimulated the secretion of ascorbate oxidase (7).

Much attention has been focused on the regulation of protein expression by metal cofactors. It is well known that metallothionein biosynthesis is inducible by the heavy metals that bind to the protein (11). The synthesis of ferritin, an iron storage protein, is also increased by iron (23). In plants, however, there have been only a few reports that the expression of protein is controlled by its prosthetic metal. Delhaize et al. (3) reported that the amount of diamine oxidase protein in clover leaves is controlled by the copper concentrations of the leaves. Jones and Carbonell (14) reported that the amount of α-amylase in aleulone layers of barley is controlled by calcium, the metal cofactor of the enzyme. In a previous paper (9), we showed that ascorbate oxidase activity in pumpkin callus could be markedly increased by adding copper, a prosthetic metal of the enzyme.

Recently, we isolated a full-length cDNA clone for ascorbate oxidase from cultured pumpkin cells, determined the nucleotide sequence of the cDNA insert, and reported that the amino acid sequence of pumpkin ascorbate oxidase is significantly homologous to those of the other blue multicopper oxidases laccase and ceruloplasmin (5).

We now report the tissue specificity of ascorbate oxidase expression in pumpkin and show that ascorbate oxidase mRNA rapidly increases during early seedling growth of pumpkin and that ascorbate oxidase mRNA in pumpkin fruit tissues is markedly induced by 2,4-D. Furthermore, we report that the marked increase in ascorbate oxidase activity in pumpkin callus brought about by the addition of copper is accompanied by an increase in ascorbate oxidase protein but not by an increase in ascorbate oxidase mRNA, suggesting that copper regulates ascorbate oxidase abundance at the translational or posttranslational level.

[1] This work was supported in part by Grants-in-Aid for Scientific Research (No. 02261214) from the Ministry of Education, Science, and Culture of Japan.

MATERIALS AND METHODS

Materials

Pumpkin (*Cucurbita* spp., Ebisu Nankin) seeds were purchased from Takii and Co., Ltd. Pumpkin plants were grown at a farm from seeds. After they were harvested, the fruit, stem, and leaf tissues were separated, frozen in liquid nitrogen, and stored at −85°C until use.

Pumpkin seeds were germinated in the dark at 25°C in dishes containing distilled water. The cotyledons were harvested at an appropriate age.

Discs (12 mm in diameter, 5 mm thick) prepared from pumpkin fruit sarcocarp tissues using a cork borer and a surgical blade under aseptic conditions were inoculated on Murashige and Skoog's basal medium with 3% sucrose and 0.8% agarose in the presence of 0, 0.01, 0.1, 1.0, 10, or 100 mg/L of 2,4-D. The material was incubated at 25°C in the dark and harvested at an appropriate age.

Pumpkin callus was induced from the fruit tissues as described previously (6) and was cultured in a 100-mL Erlenmeyer flask containing 50 mL of Murashige and Skoog's basal medium with 0 or 10 μM $CuSO_4$. The material was grown for 4, 8, or 12 d at 25°C in the dark.

Immunological Blotting

Antiserum against ascorbate oxidase was prepared as described previously (9). Electrophoresis on a 12% polyacrylamide gel containing 0.1% SDS was carried out by the method of Laemmli (16). The immunological blotting technique was performed as described by Towbin et al. (30).

Preparation of Total RNA and Northern Blot Analyses

Each tissue was ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity and the amount of protein. Total RNA was isolated from the powder by extraction with phenol/chloroform and precipitation with ethanol as described previously (5). Total RNA was electrophoresed on 1.5% (w/v) agarose gel containing 15% (v/v) formaldehyde, 20 mM Mops (pH 7.0), 8 mM sodium acetate, and 1 mM EDTA. The RNA was transferred to a Hybond N (Amersham) membrane in 10× SSC[2] (3 M NaCl, 0.3 M Na citrate), and hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) (5) at 42°C in 50% formamide as described by Maniatis et al. (19). A ^{32}P-labeled probe was prepared by random hexamer priming using [α-^{32}P]dCTP (3000 Ci·mmol^{-1}, Amersham). Hybridized blots were washed in 1× SSC and 0.1% SDS at 65°C for 1 h, followed by 0.1× SSC and 0.1% SDS at 65°C for 1 h, and then were used to expose Kodak XAR-5 x-ray film.

Assays

Ascorbate oxidase activity was assayed at 25°C by following the decrease in A_{265} of the reaction mixture containing 0.05 M potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.002% metaphosphoric acid, 0.15 mM L-ascorbic acid, and enzyme solution in a final volume of 3.0 mL, according to the method of Oberbacher and Vines (26). One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1.0 μmol of L-ascorbic acid per min.

The amount of protein was determined by the method of Bradford (2), with bovine γ-globulin as the standard.

[2] Abbreviation: SSC, standard sodium citrate.

RESULTS

Expression of Ascorbate Oxidase in Pumpkin Tissues

We investigated the amounts of ascorbate oxidase activity in various tissues of pumpkin (Fig. 1). To begin with, the specific activities of ascorbate oxidase in pumpkin seed, leaf, stem, and fruit tissues were investigated (Fig. 1B). In seeds, ascorbate oxidase activity was barely detectable. The specific ascorbate oxidase activity in leaf tissues was the highest of the four tissues and was about twice that of fruit tissues. The specific activity in stem tissues was about 1.5 times that in fruit tissues.

Total RNA prepared from each tissue was denatured, electrophoresed on an agarose gel, transferred to a nylon membrane, and hybridized to a cDNA clone (pAOP 1) for pumpkin ascorbate oxidase. In leaf and stem tissues, an abundant transcript of about 2000 nucleotides, which can be regarded as ascorbate oxidase mRNA, was observed (Fig. 1A). Fruit tissues had lower levels of ascorbate oxidase mRNA than leaf and stem tissues. In seeds, on the other hand, ascorbate oxidase mRNA was not detected. Thus, the tissue specificity of ascorbate oxidase activity was nearly in accord with that of ascorbate oxidase mRNA.

Changes in Specific Activity and mRNA of Ascorbate Oxidase during Early Seedling Growth of Pumpkin Seeds

As described above, ascorbate oxidase was abundant in leaf and stem tissues but not in dry seeds. This suggests that the expression of ascorbate oxidase is activated during seedling growth. We investigated the changes in specific activity and mRNA of ascorbate oxidase during early seedling growth of pumpkin (Fig. 2). The specific ascorbate oxidase activity in dry seeds was extremely low and nearly constant 24 h after inoculation of the seeds into dishes containing distilled water. However, the specific activity gradually increased thereafter, and the specific activity at 240 h was about 40 times that in dry seeds (Fig. 2B).

Northern blot analyses also showed that ascorbate oxidase mRNA was detected at 60 h after inoculation of the seeds into dishes containing distilled water (Fig. 2A). In the cotyledons at 60 h, which are beginning to elongate, abundant ascorbate oxidase mRNA was apparent, and thereafter the mRNA decreased. Thus, the increase in specific ascorbate oxidase activity during early seedling growth of pumpkin may be due to the increase in ascorbate oxidase mRNA.

Ascorbate Oxidase Activity during Development of Pumpkin Fruits

Figure 3 shows ascorbate oxidase activity in developing pumpkin fruits. Pumpkin fruits rapidly grew after anthesis

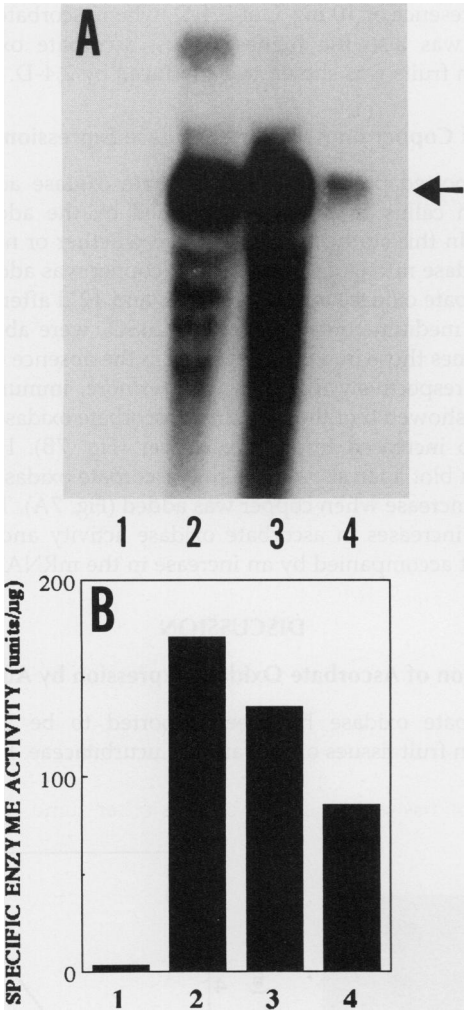

Figure 1. Expression of ascorbate oxidase in various tissues of pumpkin. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in seed (lane 1), leaf (lane 2), stem (lane 3), and fruit (lane 4). Each tissue was ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed on 1.5% (w/v) agarose gel containing 15% (v/v) formaldehyde. The RNA transferred to a membrane was hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C in 50% formamide. Arrow indicates a transcript of about 2000 nucleotides, which is regarded as mRNA for ascorbate oxidase. B, Specific activity of ascorbate oxidase in seed (lane 1), leaf (lane 2), stem (lane 3), and fruit (lane 4). Each tissue was ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assays of ascorbate oxidase activity and the amount of protein.

(Fig. 3A). The fruits at 7 d after anthesis were already large, although immature. Thereafter, the fruits gradually grew until about 30 d. Ascorbate oxidase activity in pumpkin fruits was the highest at the time of anthesis (Fig. 3B). A slow decrease in the activity was observed until 7 d after anthesis. Thereafter, the activity rapidly declined. The activity in fruits at 14 d after anthesis was one-fifteenth that at 7 d. The activity in fruits at 30 d was a little lower than that at 14 d. Thus, ascorbate oxidase activity was high in immature pumpkin fruits, which are rapidly growing.

Effect of 2,4-D on the Expression of Ascorbate Oxidase

In a previous paper (6), we reported that ascorbate oxidase activity rapidly increases during callus formation from pumpkin fruit tissues and after transfer of pumpkin callus to fresh medium. As described above, ascorbate oxidase expression is markedly activated at the time of the elongation of the cotyledons (Fig. 2). In addition, ascorbate oxidase activity in immature pumpkin fruits, which are rapidly growing, was much higher than that in mature fruits, which no longer increase in size (Fig. 3). Therefore, we investigated the changes in ascorbate oxidase activity after inoculation of pumpkin fruit tissues into Murashige and Skoog's culture medium with 3% (w/v) sucrose and 0.8% (w/v) agarose in the presence of 0 or 1.0 mg/L of 2,4-D. In the presence of 1.0 mg/L of 2,4-D, ascorbate oxidase activity markedly increased, and ascorbate oxidase activities at 2 and 4 d after

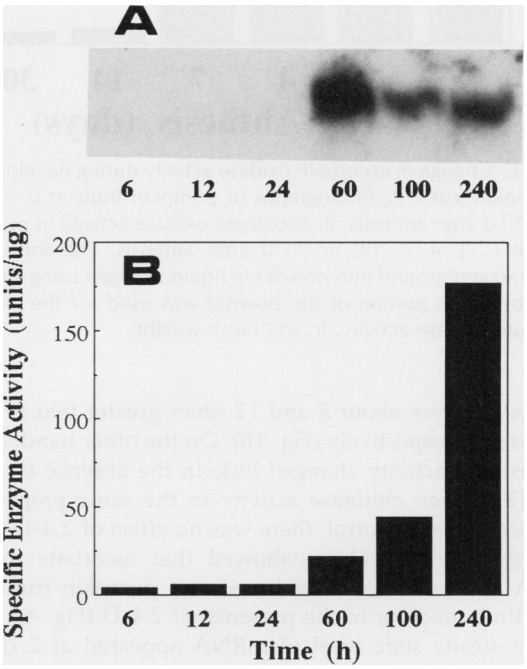

Figure 2. Changes in the activity and mRNA of ascorbate oxidase during early seedling growth of pumpkin. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in cotyledons. The seeds were germinated in the dark at 25°C in dishes containing distilled water for 6, 12, 24, 60, 100, or 240 h. The cotyledons were ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed on 1.5% (w/v) agarose gel containing 15% (v/v) formaldehyde. The RNA transferred to a membrane was hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C in 50% formamide. B, Specific activity of ascorbate oxidase in cotyledons. The seeds were germinated in the dark at 25°C in dishes containing distilled water for 6, 12, 24, 60, 100, or 240 h. The cotyledons were ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assays of ascorbate oxidase activity and the amount of protein.

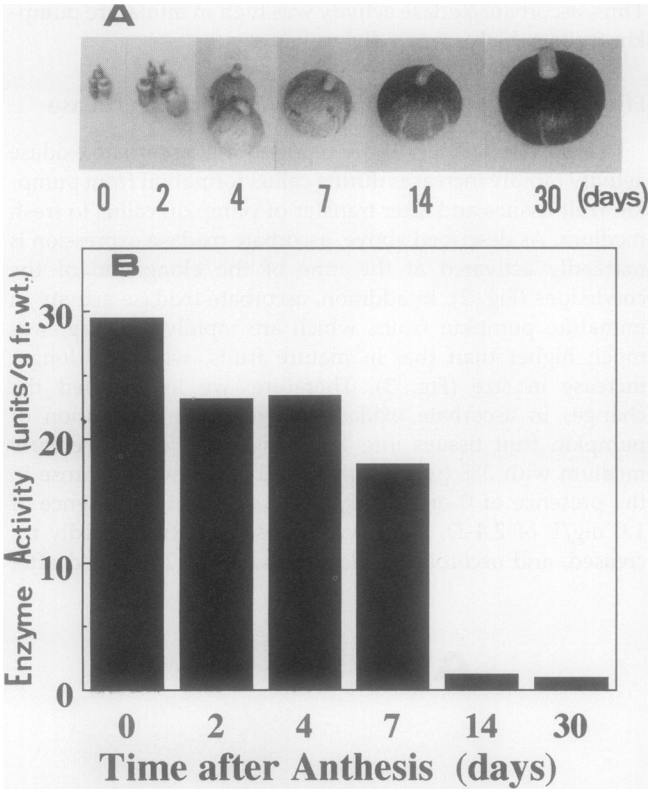

Figure 3. Change in ascorbate oxidase activity during development of pumpkin fruits. A, Photographs of pumpkin fruits at 0, 2, 4, 7, 14, or 30 d after anthesis. B, Ascorbate oxidase activity in pumpkin fruits at 0, 2, 4, 7, 14, or 30 d after anthesis. The fruits were harvested and ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity. fr. wt., Fresh weight.

inoculation were about 5 and 12 times greater that in fresh fruit tissues, respectively (Fig. 4B). On the other hand, ascorbate oxidase activity changed little in the absence of 2,4-D (Fig. 4B). When chitinase activity in the same preparation was assayed as a control, there was no effect of 2,4-D.

Northern blot analyses showed that ascorbate oxidase mRNA also increased after transfer of pumpkin fruits into the culture medium in the presence of 2,4-D (Fig. 4A). The highest steady-state level of mRNA appeared at 2 d and, thereafter, decreased (Fig. 4A). In the absence of 2,4-D, ascorbate oxidase mRNA was barely detected at 2 and 4 d after inoculation (Fig. 4A). When a more detailed time-course experiment was conducted in the presence of 1.0 mg/L of 2,4-D, ascorbate oxidase activity linearly increased after a lag phase of 1 d, and the activity at 4 d after inoculation was about 6 times that in fresh pumpkin fruits (Fig. 5B). Although ascorbate oxidase mRNA was not detected at 1 d after inoculation, it was abundant at 2 d, reached a maximum at 3 d, and subsequently decreased (Fig. 5A). Figure 6 shows the expression of ascorbate oxidase at 3 d after inoculation of pumpkin fruit tissues into the culture medium in the presence of 0, 0.01, 0.1, 1.0, 10, and 100 mg/L of 2,4-D. In the presence of 0 and 0.01 mg/L of 2,4-D, ascorbate oxidase mRNA was hardly detected. The most abundant transcript was observed in the presence of 10 mg/L of 2,4-D, when ascorbate oxidase activity was also the highest. Thus, ascorbate oxidase in pumpkin fruits was shown to be induced by 2,4-D.

Effect of Copper on Ascorbate Oxidase Expression

As reported previously (9), ascorbate oxidase activity in pumpkin callus is markedly increased by the addition of copper. In this study, we investigated whether or not ascorbate oxidase mRNA increased when copper was added.

Ascorbate oxidase activities at 4, 8, and 12 d after transfer to fresh medium containing 10 μM $CuSO_4$ were about 2, 9, and 8 times those in a culture grown in the absence of added $CuSO_4$, respectively (Fig. 7C). Furthermore, immunological blotting showed that the amount of ascorbate oxidase protein was also increased by adding copper (Fig. 7B). However, northern blot analysis showed that ascorbate oxidase mRNA did not increase when copper was added (Fig. 7A). Thus, the marked increases in ascorbate oxidase activity and protein were not accompanied by an increase in the mRNA.

DISCUSSION

Regulation of Ascorbate Oxidase Expression by Auxin

Ascorbate oxidase has been reported to be abundant mainly in fruit tissues of the family Cucurbitaceae. However,

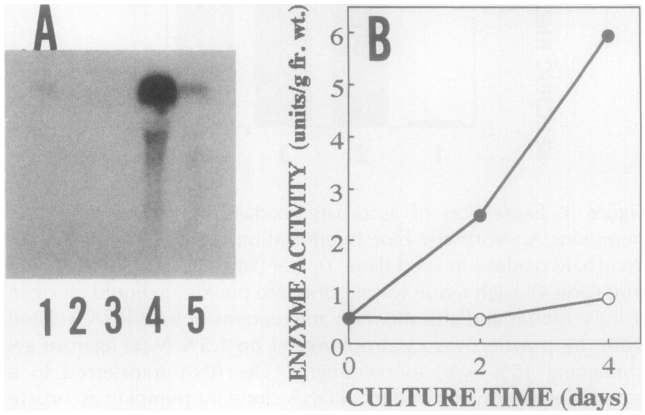

Figure 4. Effect of 2,4-D on ascorbate expression in pumpkin fruits. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 0 (lanes 1, 2, and 3) or 1.0 mg/L of 2,4-D (lanes 4 and 5) and incubated for 0 (lane 1), 2 (lanes 2 and 4), or 4 d (lanes 3 and 5) at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed, transferred to a membrane, and hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C. B, Changes in ascorbate oxidase activity in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 0 (○) or 1.0 mg/L of 2,4-D (●) and incubated for 0, 2, or 4 d at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity. fr. wt., Fresh weight.

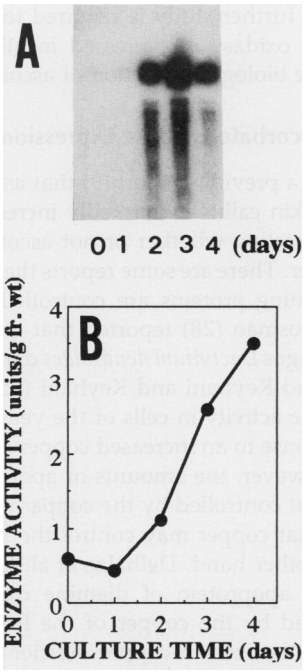

Figure 5. Changes in the activity and mRNA of ascorbate oxidase during incubation of pumpkin fruits in the presence of 1.0 mg/L of 2,4-D. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 1.0 mg/L of 2,4-D and incubated for 0, 1, 2, 3, or 4 d at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed, transferred to a membrane, and hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C. B, Changes in ascorbate oxidase activity in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 1.0 mg/L of 2,4-D and incubated for 0, 1, 2, 3, or 4 d at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity. fr. wt, Fresh weight.

fruit tissues had lower levels of ascorbate oxidase mRNA than leaf and stem tissues when the expression of ascorbate oxidase in various tissues of pumpkin was investigated (Fig. 1). In seeds, ascorbate oxidase mRNA was not detected. In cucumber, on the other hand, it has been reported that ascorbate oxidase is abundantly expressed in fruit tissues and slightly expressed in leaf, stem, and root tissues (27). At present, it is not known why the tissue specificity of ascorbate oxidase expression in pumpkin differs from that in cucumber.

In the present study, the early seedling growth of pumpkin was shown to be accompanied by the marked expression of ascorbate oxidase, which is probably caused mainly by activation at the transcriptional level (Fig. 2). It is not clear whether or not ascorbate oxidase plays an important role in early seedling growth. If it does, there is a possibility that ascorbate oxidase is involved in the elongation of cotyledons. Furthermore, ascorbate oxidase activity in immature pumpkin fruits was shown to be much higher than that in mature fruits, suggesting that ascorbate oxidase may be involved in fruit development (Fig. 3).

Finally, we showed that ascorbate oxidase in pumpkin fruits was induced by 2,4-D (Figs. 4, 5, and 6). This induction is probably controlled at the transcriptional level. Auxin is well known to stimulate stem elongation, fruit development, formation of flower buds, cell division, and cell growth and, furthermore, to induce callus formation. In particular, it is interesting that, at the cellular level, auxin has been shown to induce loosening of the cell wall, in which ascorbate oxidase may be localized (13, 22, 25). Recently, Lin and Varner (18) proposed from the study of the expression of ascorbate oxidase in zucchini that the enzyme may be involved in reorganization of the cell wall to allow for cell expansion. Our results also support the idea that ascorbate oxidase is involved in cell growth or cell division. However, it has been reported that some plants lack ascorbate oxidase activity (1). If ascorbate oxidase plays a role in cell growth and cell division, then all plants must express ascorbate

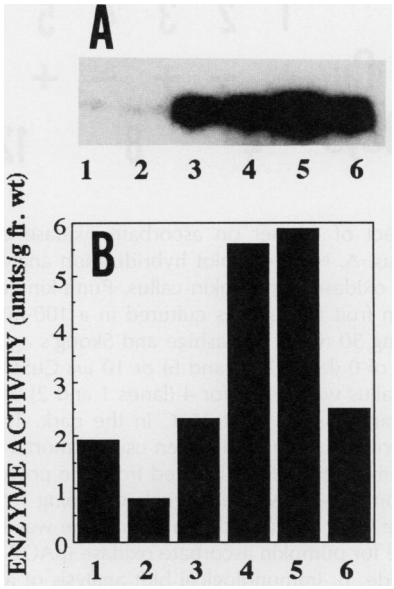

Figure 6. Effect of 2,4-D concentration on ascorbate oxidase expression after incubation of pumpkin fruits for 3 d. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), 1.0 (lane 4), 10 (lane 5), or 100 mg/L of 2,4-D (lane 6) and incubated for 3 d at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed, transferred to a membrane, and hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C. B, Ascorbate oxidase activity in pumpkin fruits. Discs prepared from pumpkin fruit sarcocarp tissues were inoculated on Murashige and Skoog's agar medium in the presence of 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), 1.0 (lane 4), 10 (lane 5), or 100 mg/L of 2,4-D (lane 6) and incubated for 3 d at 25°C in the dark. The discs were ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity. fr. wt, Fresh weight.

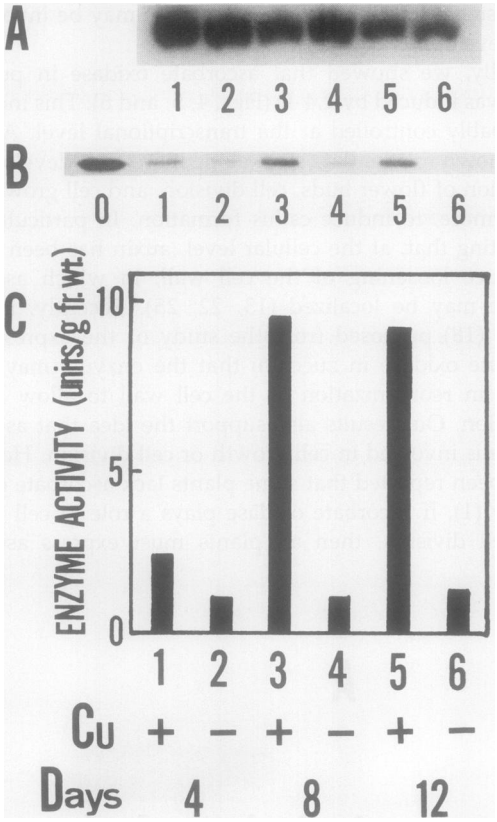

Figure 7. Effect of copper on ascorbate oxidase expression in pumpkin callus. A, Northern blot hybridization analysis of mRNA for ascorbate oxidase in pumpkin callus. Pumpkin callus induced from pumpkin fruit tissues was cultured in a 100-mL Erlenmeyer flask containing 50 mL of Murashige and Skoog's agar medium in the presence of 0 (lanes 2, 4, and 6) or 10 μM $CuSO_4$ (lanes 1, 3, and 5). The callus was grown for 4 (lanes 1 and 2), 8 (lanes 3 and 4), or 12 d (lanes 5 and 6) at 25°C in the dark. The callus was ground into powder in liquid nitrogen using a mortar and a pestle. Fifty micrograms of total RNA isolated from the powder was electrophoresed on 1.5% (w/v) agarose gel containing 15% (v/v) formaldehyde. The RNA transferred to a membrane was hybridized to a cDNA clone for pumpkin ascorbate oxidase (pAOP 1) at 42°C in 50% formamide. B, Immunological blot analysis of ascorbate oxidase protein in pumpkin callus with antiserum against ascorbate oxidase. Pumpkin callus induced from pumpkin fruit tissues was cultured in a 100-mL Erlenmeyer flask containing 50 mL of Murashige and Skoog's agar medium in the presence of 0 (lanes 2, 4, and 6) or 10 μM $CuSO_4$ (lanes 1, 3, and 5). The callus was grown for 4 (lanes 1 and 2), 8 (lanes 3 and 4), or 12 d (lanes 5 and 6) at 25°C in the dark. The callus was ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder and purified ascorbate oxidase (lane 0) were used for the immunological blot analysis with antiserum against ascorbate oxidase. C, Ascorbate oxidase activity in pumpkin callus. Pumpkin callus induced from pumpkin fruit tissues was cultured in a 100-mL Erlenmeyer flask containing 50 mL of Murashige and Skoog's agar medium in the presence of 0 (lanes 2, 4, and 6) or 10 μM $CuSO_4$ (lanes 1, 3, and 5). The callus was grown for 4 (lanes 1 and 2), 8 (lanes 3 and 4), or 12 d (lanes 5 and 6) at 25°C in the dark. The callus was ground into powder in liquid nitrogen using a mortar and a pestle. A portion of the powder was used for the assay of ascorbate oxidase activity. fr. wt., Fresh weight.

oxidase. Clearly, further study is required to show whether or not ascorbate oxidase is expressed in all plants and to clarify the precise biological function of ascorbate oxidase.

Regulation of Ascorbate Oxidase Expression by Copper

We showed in a previous report (9) that ascorbate oxidase activity in pumpkin callus is markedly increased by adding copper. It is interesting whether or not ascorbate oxidase is induced by copper. There are some reports that the expression of copper-containing proteins are controlled by the metal. Shatzman and Kosman (28) reported that galactose oxidase activity in the fungus *Dactylium dendroides* depends on copper concentration, and Keyhani and Keyhani (15) also reported that Cyt *c* oxidase activity in cells of the yeast *Candida utilis* increases in response to an increased copper concentration in the medium. However, the amounts of apoproteins of these enzymes were not controlled by the copper concentration. It was suggested that copper may control the holoenzyme assembly. On the other hand, Delhalze et al. (3) reported that the synthesis of apoprotein of diamine oxidase in clover leaves is regulated by the copper of the leaves so that no apoprotein is synthesized in copper-deficient leaves. Metallothionein, a metal-binding protein, is well known to be induced by a heavy metal such as copper. In this case, copper activates metallothionein gene transcription by a specific DNA-binding protein (10). Furthermore, copper-dependent gene expression of Cyt c_{552} synthesis in *Chlamydomonas reinhardtii* was directly affected by copper concentration (21).

In the present study, we showed that the amount of mRNA coding for ascorbate oxidase was not affected by copper, although the amount of ascorbate oxidase protein was increased (Fig. 7). We suggest that copper controls ascorbate oxidase expression at the translational or posttranslational level. One possibility is that the translation of ascorbate oxidase mRNA is specifically stimulated by copper. For example, the expression of ferritin protein is increased by iron. This regulation is achieved primarily by increased translation of existing mRNA coding for ferritin (12). Another possibility is that copper helps to stabilize ascorbate oxidase proteins and protects them from the proteolytic breakdown. At present, we have no experimental results supporting either hypothesis. Further study will be required to clarify the mechanism of control of ascorbate oxidase expression by copper.

ACKNOWLEDGMENTS

The authors wish to express their sincere gratitude to Professor A. Shinmyo, Osaka University, and Professor T. Hattori, Mie University, Japan, for their kind suggestions and useful discussion.

LITERATURE CITED

1. **Arrigoni O, Dipierro S, Borraccino G** (1981) Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. FEBS Lett **125:** 242–244
2. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
3. **Delhaize E, Dilworth MJ, Webb J** (1986) The effects of copper nutrition and developmental state on the biosynthesis of diamine oxidase in clover leaves. Plant Physiol **82:** 1126–1131
4. **Esaka M, Fukui H, Suzuki K, Kubota K** (1989) Secretion of

ascorbate oxidase by suspension-cultured pumpkin cells. Phytochemistry **28:** 117–119

5. **Esaka M, Hattori T, Fujisawa K, Sakajo S, Asahi T** (1990) Molecular cloning and nucleotide sequence of full-length cDNA for ascorbate oxidase from cultured pumpkin cells. Eur J Biochem **191:** 537–541
6. **Esaka M, Imagi J, Suzuki K, Kubota K** (1988) Formation of ascorbate oxidase in cultured pumpkin cells. Plant Cell Physiol **29:** 231–235
7. **Esaka M, Nishitani I, Fukui H, Suzuki K, Kubota K** (1989) Stimulation of ascorbate oxidase secretion from cultured pumpkin cells by divalent cations. Phytochemistry **28:** 2655–2658
8. **Esaka M, Suzuki K, Kubota K** (1985) Determination method for L-ascorbic acid in foods with immobilized ascorbate oxidase. Agric Biol Chem **49:** 2955–2960
9. **Esaka M, Uchida M, Fukui H, Kubota K, Suzuki K** (1988) Marked increase in ascorbate oxidase protein in pumpkin callus by adding copper. Plant Physiol **88:** 656–660
10. **Fürst P, Hu S, Hackett R, Hamer D** (1988) Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. Cell **55:** 705–717
11. **Hamer DH** (1986) Metallothionein. Annu Rev Biochem **55:** 913–951
12. **Harrell CM, McKenzie AR, Patino MM, Walden WE, Theil EC** (1991) Ferritin mRNA: interactions of iron regulatory element with translational regulator protein P-90 and the effect on base-paired flanking regions. Proc Natl Acad Sci USA **88:** 4166–4170
13. **Honda SI** (1955) Ascorbic acid oxidase in barley roots. Plant Physiol **30:** 174–181
14. **Jones RL, Carbonell J** (1984) Regulation of the synthesis of barley aleurone α-amylase by gibberellic acid and calcium ions. Plant Physiol **76:** 213–218
15. **Keyhani E, Keyhani J** (1975) Cytochrome *c* oxidase biosynthesis and assembly in *Candida utilis* yeast cells. Arch Biochem Biophys **167:** 596–602
16. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
17. **Lee MH, Dawson CR** (1973) Ascorbate oxidase: further studies on the purification of the enzyme. J Biol Chem **248:** 6596–6602
18. **Lin LS, Varner JE** (1991) Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.) Plant Physiol **96:** 159–165
19. **Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. **Matsumoto K, Yamada K, Osajima Y** (1981) Ascorbate electrode for determination of L-ascorbic acid in food. Anal Chem **53:** 1974–1979
21. **Merchant S, Bogorad L** (1987) Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552. EMBO J **6:** 2531–2535
22. **Mertz D** (1961) Distribution and cellular localization of ascorbic acid oxidase in the maize root tip. Am J Bot **48:** 405–413
23. **Miller LL, Miller SC, Torti SV, Tsuji Y, Torti FM** (1991) Iron-independent induction of ferritin H chain by tumor necrosis factor. Proc Natl Acad Sci USA **88:** 4946–4950
24. **Nakamura T, Makino N, Ogura Y** (1963) Purification and properties of ascorbate oxidase from cucumber. J Biochem **64:** 189–195
25. **Newcomb EH** (1951) Effect of auxin on ascorbic oxidase activity in tobacco pith cells. Proc Soc Exp Biol Med **76:** 504–509
26. **Oberbacher MF, Vines HM** (1963) Spectrophotometric assay of ascorbic acid oxidase. Nature **197:** 1203–1204
27. **Ohkawa J, Okada N, Shinmyo A, Takano M** (1989) Primary structure of cucumber (*Cucumis sativus*) ascorbate oxidase deduced from cDNA sequence: homology with blue copper proteins and tissue-specific expression. Proc Natl Acad Sci USA **86:** 1239–1243
28. **Shatzman AR, Kosman DJ** (1978) The utilization of copper and its role in the biosynthesis of copper-containing proteins in fungus *Dactylium dendroides*. Biochim Biophys Acta **544:** 163–179
29. **Stark GR, Dawson CR** (1962) On the accessibility of sulfhydryl groups in ascorbic acid oxidase. J Biol Chem **237:** 712–716
30. **Towbin H, Staehelin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354
31. **Vines HM, Oberbacher MF** (1963) Citrus fruit enzymes. 1. Ascorbic acid oxidase in orange. Plant Physiol **38:** 333–337
32. **Weis W** (1975) Ascorbic acid and electron transport. Ann NY Acad Sci **258:** 190–200
33. **Yamauchi H, Yamawaki Y, Ueda Y, Chachin K** (1984) Subcellular localization of redox enzymes involving ascorbic acid in cucumber fruits. J Jpn Soc Hort Sci **53:** 347–353