Localization of Target-Site of the Protoporphyrinogen Oxidase-Inhibiting Herbicide, S-23142, in *Spinacia oleracea* L.

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Z. Naturforsch. **48c**, 350–355 (1993); received November 16, 1992

ALA, *Nicotiana tabacum*, Photomixotrophic Cell, Protoporphyrin IX, Protoporphyrinogen Oxidase Inhibition, Aminolevulinic Acid

Effects of S-23142 on protoporphyrin IX (Proto IX) biosynthesis in chloroplasts isolated from *Spinacia oleracea* L. were examined using reverse phase HPLC with fluorescence monitoring. The synthesis of Proto IX was inhibited to a level of 50% by 10^{-9} M of S-23142 in this system. The effects of S-23142 was also tested in chloroplasts isolated from two types of photomixotrophic tobacco cells, wild type and S-23142 tolerant cells. The biosynthesis of both the wild type cells and YZI-1 S cells were inhibited at 50% by 10^{-9} M and 10^{-7} M of S-23142, respectively. It is, therefore evident that the mutation in the tolerant cell is associated with the Protox. To investigate the localization of Proto IX biosynthesis and the target site of S-23142, spinach chloroplasts were osmotically broken and separated into stroma and membrane (thylakoid and envelope) fractions. A very active Proto IX synthesis from ALA was found in the stromal fraction, while no activity of Proto IX synthesis was observed in the membrane fraction. These results suggest that most Proto IX synthetic activity and a target-site of S-23142 exist in the stromal fraction.

Introduction

Phthalimide compounds such as S-23142 (N-[4-chloro-2-fluoro-5-propagyloxy]-phenyl-3,4,5,6-tetrahydrophthalimide) have a very similar mode of action to diphenyl ether compounds such as acifluorfen (AF) despite of the difference in their chemical structures ([1], see Fig. 1). These herbicides markedly increase the intracellular levels of the photodynamic tetrapyrrole, protoporphyrin IX (Proto IX), causing light dependent phytotoxic effects [2–4]. The herbicidal action of these chemicals has been attributed to the accumulation of Proto IX which is a strong phytotoxic photosensitizer. Herbicides of this type also inhibit protoporphyrinogen oxidase (Protox) which is the terminal enzyme of the common branch of the heme and Chl biosynthetic pathways in plants and oxidizes protoporphyrinogen to protoporphyrin [5–7]. Accordingly, phthalimide and diphenyl ether type herbicides are known as Protox-inhibiting herbicides.

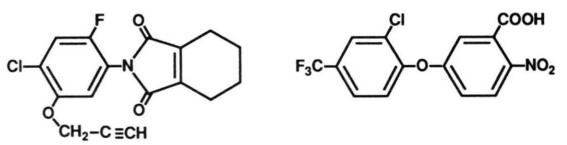

Fig. 1. Chemical structure of S-23142 and acifluorfen (AF).

Characterization and localization of the target site of the herbicides are important steps in the mode of action studies of Protox-inhibiting herbicides. Recently, Matringe *et al.* reported that the diphenyl ether type herbicides such as acifluorfen bind to corn etioplast membranes and that this binding of herbicides is competitively inhibited by Proto IX [8]. In addition, they reported that the diphenyl ether herbicides inhibit Protox activity, but not the activity of Mg-chelatase, and also proposed Protox as a cellular target site for diphenyl

Abbreviations: AFE, acifluorfen-ethyl; ALA, δ-aminolevulinic acid; Copro III, coproporphyrin III; Coprogen, coproporphyrinogen III; Proto IX, protoporphyrin IX; Protogen, protoporphyrinogen IX; Protox, protoporphyrinogen oxidase.

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0939–5075/93/0300–0350 $ 01.30/0

ether herbicides. Phthalimide compounds such as S-23142 exhibited the inhibition of Protox and the binding to a solubilized plastid fraction in a similar manner as diphenyl ether herbicide [9]. These results suggests that target site of S-23142 is also Protox [9]. However, characterization and localization of the target site of Protox-inhibiting herbicides are still not known in detail. For further understanding of the target site, it is necessary to determine whether isolated chloroplasts can carry out the herbicide sensitive-porphyrin synthesis and whether this herbicidal activity can be detected in isolated chloroplast membranes. The aims of this study were thus to identify the localization of porphyrin formation activity, to detect the sensitivity to Protox-inhibiting herbicides in each part of chloroplasts and also to discuss characterization of the target site of Protox-inhibiting herbicides.

Materials and Methods

Plant materials

Fresh leaves of spinach (*Spinacia oleracea* L.) were harvested and, in the absence of light, were kept immersed in water for 2 days. The leaves were subsequently irradiated with light (1.8 W/m^2, 25 °C) before extraction of chloroplasts.

Cell culture

A culture of photomixotrophic tobacco (*Nicotiana tabacum* L. *cv.* Samsun NN) cells was used and was maintained in modified Linsmaier-Skoog basal medium [10] with twice the original level of vitamins, 10^{-5} M 1-naphthaleneacetic acid (NAA), 10^{-6} M kinetin (6-furfurylaminopurine) and 3% sucrose as described before [11, 12]. The photomixotrophic cultured cells were grown in 200 ml Erlenmeyer flasks and continuously agitated on a reciprocal shaker (120 rpm; NR-10, Taitec, Tokyo) and kept at 26 ± 2 °C in the light (approx. 30 W/m^{-2}).

Isolation of intact chloroplasts

Spinach chloroplasts were isolated by the method of Mills and Joy [13] with slight modifications. The leaves (50 g) were cut into small pieces, directly immersed in 150 ml icecold chloroplast extraction medium (330 mM sorbitol, 50 mM Tricine [N-Tris(hydroxymethyl)methylglycine]-KOH, pH 7.9, 2 mM EDTA, 1 mM $MgCl_2$, and 0.1% BSA) and homogenized for 5 s with a Polytron (PT 10/35; Kinematica, Basel, Switzerland). After filtration through four layers of gauze, 30 ml of the homogenate was poured into each 50 ml centrifuge tube and was underlayered with 14 ml of Percoll medium (40% (v/v) Percoll, 330 mM sorbitol, 50 mM Tricine, pH 7.9, and 0.1% BSA). Chloroplasts were peletted by centrifugation at 2,500 × *g* for 1 min in a LC-121 centrifuge (Tomy Seiko Co., Ltd., Tokyo, Japan) equipped with a TS-7 swing-out rotor. The supernatant, and then the percoll layer, were removed by aspiration and the pellet gently resuspended in appropriate medium with a small piece of nylon mesh attached to a glass rod. The entire isolation procedure could be accomplished in less than 5 min. The resulting suspension usually contained 90–95% intact chloroplasts measured by ferricyanide reduction [14]. Photomixotrophic cultured tobacco cells were also homogenized in the same chloroplast extraction medium with a hand homogenizer. The homogenate was filtrated with four layers of gauze, and the chloroplasts were pelleted by centrifuging at 2,500 × *g* for 30 s. Following resuspension in 50 ml chloroplast extraction medium, and centrifugation at 2,500 × *g* for 30 s, the chloroplasts were resuspended in 1 ml of the same medium.

Preparation of stromal extracts

Chloroplast stromal extract was prepared by the method of Furbank and Lilley [15]. For the preparation of chloroplast extract, isolated chloroplasts were osmotically shocked by resuspension in a 1/13 dilution of chloroplast extraction medium and centrifuged for 15 min at 9,000 × *g*. The resulting pellet of envelope-free chloroplasts was retained for Chl estimation [22]. The supernatant (chloroplast stromal extract) was subjected to concentration by ultra filtration using a Centricon 10 (Amicon Grace Co. Ltd., MA, U.S.A.). The concentrated chloroplast extract was immediately subdivided into 250 µl aliquots into small serum tubes and placed in liquid N_2. Assays on a rethawed sample of chloroplast extract were completed within 3 h. Activity loss under these conditions was less than 1% per day for all measured activities. Protein contents were determined by the method of Bradford [16] using BSA as a standard.

Measurement of protoporphyrin synthesizing activities

Assay using ALA: Proto IX synthesis from ALA was measured using the procedure of Prado *et al.* [17] and Mito *et al.* [9]. Proto IX synthesizing activities of the intact chloroplasts, the stromal extracts and the membrane extracts were measured using ALA as the substrate. Each chloroplast fraction (the intact chloroplasts, the stromal extracts and the membrane extracts; equivalent to 1 to 2 mg protein) was added to 500 µl of a reaction medium (4 mM ALA, 0.5 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, 10 mM Hepes-pH 7.5, 4 mM glutathione, 0.6 mM NAD, 1.5 mM ATP, 2% BSA). The mixture was incubated for 3 h at 25 °C in darkness, and the reaction was stopped by addition of 1 ml of methanol. After centrifugation at 10,000 × *g* for 5 min, synthesized Proto IX was measured by HPLC.

Assay using Coprogen

This was undertaken using the direct spectrofluorometric assay described previously [18, 19]. Stromal extracts were suspended in 500 µl of a reaction medium containing 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT. The reaction was started by addition of 40 ml of a solution containing 6 nmol of coproporphyrinogen (Coprogen) in 0.25 M Tris-HCl, pH 7.5, containing 25 mM DTT. The Proto IX production was fluorometrically monitored with CytoFluor 2300 (Millipore Co. MA, U.S.A.) at λex = 410 nm and λem = 640 nm [20].

HPLC analysis

To analyze Proto IX in the reaction mixture, a C_8 reversed phase column (Senshu Pak, C8-2251-N, 6 mm × 250 mm, Senshu Science Co., Ltd., Tokyo, Japan) was used with a solvent system of 85% methanol containing 10 mM ammonium acetate (pH 6.0), under a flow rate of 1 ml/min. Proto IX detection was performed with a fluorescence detector (F-1000, Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelength settings at 420 and 630 nm, respectively.

Chemicals

Coprogen was prepared from coproporphyrin III (Porphyrin Products, Logan, UT) by reduction with sodium amalgam as described previously [21]. The reduced porphyrin solution was neutralized by dilution into an equal volume of the Tris buffer plus DTT solution as described previously for the direct assay for Protox. Herbicide; S-23142 was kindly offered by Sumitomo Chemical Co. Ltd., and AF was offered by Dr. Yoneyama of Utsunomiya Univ. Other chemicals were purchased from Nakaraitesk Co., Ltd., Kyoto, Japan.

Results

Porphyrin synthesis from ALA in isolated chloroplasts

Porphyrins biosynthesized from ALA in isolated chloroplasts were analyzed by HPLC and the effect of Protox-inhibiting herbicides on the protoporphyrin biosynthesis was examined. Synthesized porphyrins were separated using a reverse phase column and detected by a fluorescence monitor at 640 nm, and one significant peak and three minor peaks were detected (Fig. 2). The major peak (retention time 23 min) was assigned to be Proto IX by comparison of its retention time and fluorescence spectra with those of the authentic sample. However, the other minor peaks could not be identified. Synthesis of Proto IX was inhibited by 10^{-6} M of S-23142. Fig. 3 shows the dose-dependent inhibition of Proto IX by S-23142. S-23142 inhibited Proto IX synthesis by 50% at 10^{-9} M, when ALA was given as the start precursor.

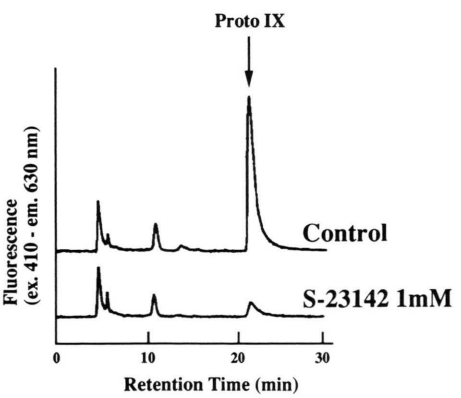

Fig. 2. HPLC profiles on porphyrins synthesized by intact chloroplasts. Porphyrin in the reaction mixture were separated by reverse phase HPLC as described in the Materials and Methods section. Detection was by fluorescence (excitation, 410 nm; emission, 630 nm). This experiment was done three times with similar results.

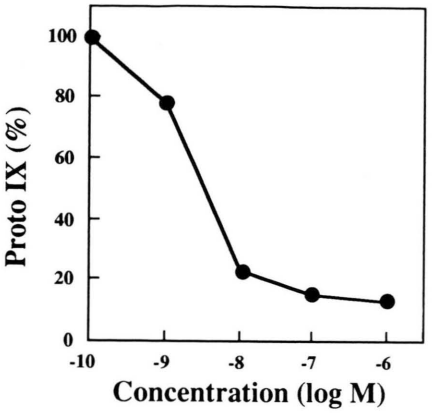

Fig. 3. Dose-dependent inhibition Proto IX synthesis from ALA in intact chloroplasts by S-23142 (●). The Proto IX content is given as a relative value (%), calculated with reference to the control rate. Specific activity of the control was 0.05 nmol mg protein^{-1} h^{-1}. The values represent the average of three independent measurements.

Porphyrin synthesis from ALA in intact chloroplasts of photomixotrophic tobacco cells

Photomixotrophic cultured cells of tobacco have developed chloroplasts involving the ability of photosynthesis and the pathway of chlorophyll synthesis. Recently, the S-23142 tolerant photomixotrophic tobacco cells named YZI-1 S were selected and it was suggested that the variation causing herbicide tolerance should be either genetic or epigenetic rather than an induced physiological response to herbicide exposure and also that the mutation should occur in the target site and/or the near site (will be submitted elsewhere). Following this, the porphyrins synthesized from ALA in isolated chloroplasts from XZI-1 S and wild type cells were analyzed by reverse phase HPLC, several porphyrins including Proto IX were detected. The Proto IX biosynthesis in both the wild type and YZI-1 S cells was inhibited by S-23142, and a sigmoidal relationship was found between the S-23142 concentration and Proto IX accumulation in both cell lines (Fig. 4). The Proto IX biosyntheses in the wild type and the tolerant cells were inhibited at 50% by 10^{-9} M and 10^{-7} M of S-23142, respectively. Since the YZI-1 S cells exhibited tolerance in cell bleaching by 100–200 times high concentration of S-23142 than that for the wild cells, this gap of I_{50} values in Proto IX accumulation seems reasonable.

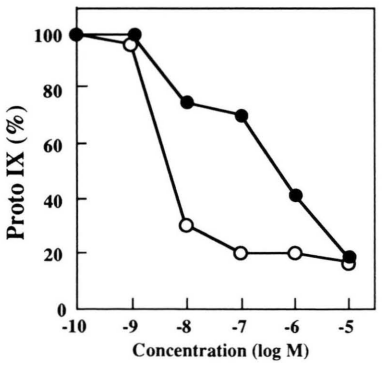

Fig. 4. Dose-dependent inhibition Proto IX synthesis from ALA in photomixotrophic tobacco wild type cells (○) and YZI-1 S (●) cells by S-23142. The Proto IX content is given as a relative value (%), calculated with reference to rate in each control experiment. Each control activity of wild type cell and YZI-1 S cell was 5 and 1.5 pmol mg protein^{-1} h^{-1}, respectively. The values represent the average of three independent measurements.

Proto IX synthesis in each part of chloroplasts

To investigate localization of Proto IX biosynthesis, chloroplasts isolated from spinach leaves were osmotically broken and separated into stromal and membrane (thylakoid and envelope) fractions, and Proto IX synthetic activity from ALA was subsequently measured in the stromal and the membrane fractions. A very active Proto IX synthesis was found in the stromal fraction, while no activity of Proto IX synthesis was observed in membrane fractions (Table I). This Proto IX synthetic activity in stromal extracts was ob-

Table I. Localization of Proto IX synthetic activity in different parts of the chloroplast, stromal extract and membrane fraction.

Part of chloroplasts	Proto IX content [%][a]
Stromal extract	92
Membrane fraction	11
Stromal extract and membrane fraction	98
Chloroplasts	100[b]

[a] The Proto IX content is given as a relative value (%), calculated with reference to experimental rate in chloroplasts. The values represent the averages of three measurements.

[b] Specific activity of Proto IX synthesis in chloroplasts was 0.05 nmol mg protein^{-1} h^{-1}.

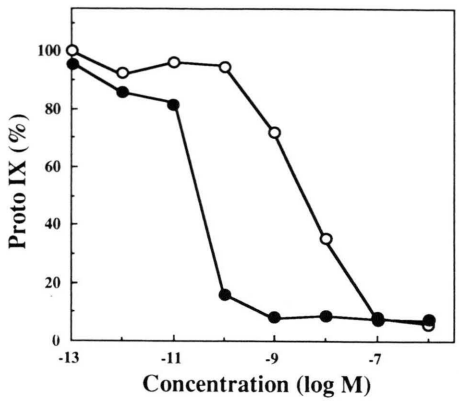

Fig. 5. Dose-dependent inhibition Proto IX synthesis from ALA in stromal extracts by S-23142 (●) and AF (○). The Proto IX content is given as a relative value (%), calculated with reference to the control rate. Specific activity of the control was 0.04 nmol mg protein^{-1} h^{-1}. The values represent the average of three independent measurements.

served even after ultracentrifugation (100,000 × *g*, 1 h), and this fact suggests that the biosynthesis is carried out in solubilized stromal extracts (data not shown). AF also inhibited the Proto IX synthesis from ALA and the dose-dependent inhibition by these herbicides is shown in Fig. 5. Half inhibition values (I_{50}) for S-23142 and AF in stromal extracts were roughly 5×10^{-11} M and 5×10^{-9} M, respectively.

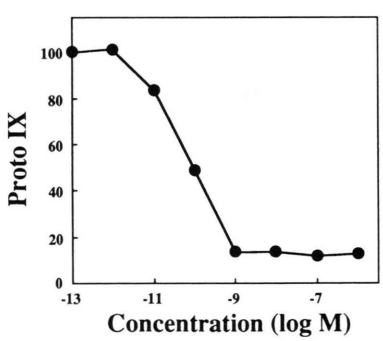

Fig. 6. Dose-dependent inhibition Proto IX synthesis from Coprogen in stromal extracts by S-23142. The Proto IX content is given as a relative value (%), calculated with reference to the rate in each control experiment. Specific activity of the control was 2.1 nmol mg protein^{-1} h^{-1}. The values represent the average of three independent measurements.

Proto IX synthesis from Coprogen

Since Proto IX was synthesized from Coprogen in two steps, Coprogen–Protogen–Proto IX, Coprogen would be a suitable substrate to analyze the Proto IX synthetic activity. The stromal fractions effectively converted Coprogen to Proto IX, and this conversion was inhibited by S-23142 in a dose-dependent manner (Fig. 6).

Discussion

It has been reported that Proto IX is synthesized from ALA in intact corn plastids and in solubilized plastid membranes [9, 18]. An inhibition of Proto IX synthetic activity by Protox-inhibiting herbicides and binding of S-23142 [9] to corn solubilized plastid membranes and of AF to corn etioplast membranes [8] have also been reported. It was proposed that synthetic enzymes of ALA to Proto IX exist on the plastid membranes and Protox-inhibiting herbicides bind to the target site (Protox) of the membranes.

In our experiments, Proto IX synthesis from ALA were detected in stromal extracts (soluble fraction of chloroplasts), and was inhibited by treatment of S-23142 and AF. On the other hand, the Proto IX synthesis in the membranes fraction of chloroplasts was not detected. These results suggest that the Proto IX biosynthetic pathway from ALA should exist in stromal fraction, and a target-site of Protox-inhibiting herbicides also locates in the stromal fraction of chloroplasts.

There are several possible interpretations for difference in localization of the Proto IX synthetic activity and of the target site in etioplasts and chloroplasts. For example, localization of the Proto IX biosynthesis pathway might differ between corn etioplasts and spinach chloroplasts or the porphyrin biosynthetic system on the membranes might be solubilized during the procedure for the separation of stroma and membrane. The cause of the different results to explain localization of Protox is not clear yet, however further investigation such as a binding study of Protox-inhibiting herbicides with stromal fraction will give better understanding for this fact.

In our experiments, Proto IX accumulation in both intact chloroplasts and stromal extracts were decreased by Protox-inhibiting herbicides only when ALA was given as the substrate. Recently,

the effect of acifluorfen-Na and S-23142 on the *in vitro* synthesis of Proto IX by isolated plastids was reported that no Proto IX accumulation was observed *in vitro* [9, 23]. On the other hand, Protox-inhibiting herbicides greatly increased the intracellular levels of Proto IX in whole plants and detached leaves, and then caused light dependent phytotoxic effects [2–4]. The reason of decreasing of the Proto IX accumulation in isolated chloroplasts and etioplasts by Protox-inhibiting herbicides is highly interesting. Jacobs *et al.* [19] suggested that accumulated Protogen by the herbicide-inhibition can be oxidized by a Protogen oxidizing system that is located at sites such as the plasma membrane, which was much less sensitive to the herbicidal action in our assay. This suggestion possibly is an answer why protogen synthesized from ALA in the isolated chloroplasts and the stromal fractions could not be oxidized to Proto IX because of lacking the plasma membrane in our reaction mixture.

The Proto IX synthesis was also observed in chloroplasts isolated from the photomixotrophic cultured wild type and S-23142 tolerant cells. The Proto IX synthesis in wild type cells was inhibited so a level of 50% by 10^{-9} M of S-23142, while that in the tolerant cells was inhibited to a level of 10^{-7} M. It is, therefore evident that tolerance mutation in YZI-1S cells is expressed in chloroplasts and is associated with the target site of Protox-inhibiting herbicides. This YZI-1S cell line is considered to be a very useful material for elucidating the mode of action of Protox-inhibiting herbicides. Molecular mechanisms of Protox-inhibiting herbicides using this cell line are in progress.

Acknowledgements

We wish to express our thanks to Sumitomo Chemical Co. Ltd. for S-23142 and Dr. Koichi Yoneyama of Utsunomiya University for AF. We would like to thank Mr. Tae-Jun Kim of the Korea Research Institute of Chemical Technology (KRICT) for experimental cooperation. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

[1] R. Sato, E. Nagano, H. Oshio, and K. Kamoshita, Pestic. Biochem. Physiol. **28,** 194–200 (1987).
[2] J. M. Becerril and S. O. Duke, Plant Physiol. **90,** 1175–1181 (1989).
[3] J. R. Bowyer, B. J. Hallahan, P. Camilleri, and J. Howard, Plant Physiol. **89,** 674–680 (1989).
[4] S. O. Duke, J. Lydon, and R. N. Paul, Weed Sci. **37,** 152–160 (1989).
[5] M. Matringe, J. M. Camadro, P. Labbe, and R. Scalla, Biochem. J. **260,** 231–235 (1989).
[6] J. M. Camadro, M. Matringe, R. Scalla, and P. Labbe, Biochem. J. **277,** 17–21 (1991).
[7] J. M. Jacobs, N. J. Jacobs, S. E. Borotz, and M. L. Guerinot, Arch. Biochem. Biophys. **280,** 369–375 (1990).
[8] R. Varsano, M. Matringe, N. Magnin, R. Mornet, and R. Scalla, FEBS Lett. **272,** 106–108 (1990).
[9] N. Mito, R. Sato, M. Miyakado, H. Oshio, and S. Tanaka, Pestic. Biochem. Physiol. **40,** 128–135 (1991).
[10] E. M. Linsmaier and F. Skoog, Physiol. Plant. **18,** 100–127 (1965).
[11] F. Sato, S. Takeda, and Y. Yamada, Plant Cell Reports **6,** 401–404 (1987).
[12] F. Sato, K. Asada, and Y. Yamada, Plant Cell Physiol. **20,** 193–200 (1979).
[13] W. R. Mills and K. W. Joy, Planta **148,** 75–83 (1980).
[14] R. McC. Lilley, M. P. Fitzgerald, K. G. Rienits, and D. A. Walker, New Phytol. **75,** 1–10 (1975).
[15] R. T. Furbank and R. McC. Lilley, Plant Physiol. **67,** 1036–1041 (1981).
[16] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[17] A. D. Prado, B. M. Chereskin, P. A. Castelfranco, V. R. Franceshi, and B. E. Wezelman, Plant Physiol. **65,** 956 (1980).
[18] P. Labbe, J. M. Camadro, and H. Chambon, Anal. Biochem. **149,** 248–260 (1985).
[19] J. M. Jacobs, N. J. Jacobs, T. D. Sherman, and S. O. Duke, Plant Physiol. **97,** 197–203 (1991).
[20] P. Labbe, J. M. Camadro, and H. Chambon, Anal. Biochem. **149,** 248–260 (1985).
[21] J. M. Jacobs, N. J. Jacobs, S. E. Borotz, and M. L. Guerinot, Arch. Biochem. Biophys. **280,** 369–375 (1990).
[22] D. I. Arnon, Plant Physiol. **24,** 1–15 (1949).
[23] C. A. Rebeiz, K. N. Reddy, V. B. Nandihalli, and J. Velu, Photochem. Photobiol. **52,** 1099–1117 (1990).