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Physiology and Growth of Transgenic Tobacco Plants Containing *Bacillus subtilis* Protoporphyrinogen Oxidase Gene in Response to Oxyfluorfen Treatment

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Bacillus subtilis Protoporphyrinogen Oxidase 유전자 형질전환 담배의 Oxyfluorfen 처리에 대한 생리 · 생장반응

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ABSTRACT

The transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants containing *Bacillus subtilis* protoporphyrinogen oxidase gene with cauliflower mosaic virus 35S promoter have recently been generated by using *Agrobacterium*-mediated gene transformation. The nontransgenic and the transgenic tobacco plants were compared with respect to responses to diphenyl ether herbicide oxyfluorfen and under various environmental conditions. Both cellular leakage and lipid peroxidation caused by oxyfluorfen were found to be less in the transgenic than in the nontransgenic plants. Growth responses of the transgenic plants under various temperature, light, and water conditions were almost the same as those of the nontransgenic plants, although the transgenic plants exhibited slightly more retarded growth under low light or saturated water condition. These results revealed that the transgenic tobacco plants containing *B. subtilis* protoporphyrinogen oxidase gene under cauliflower mosaic virus 35S promoter were relatively resistant to oxyfluorfen and exhibited normal growth pattern. Possible mechanism of resistance to oxyfluorfen in the transgenic plants is also discussed.

Key words : *Bacillus subtilis*, Diphenyl ether herbicides, Oxyfluorfen, Protoporphyrinogen oxidase, Transgenic tobacco plants.

INTRODUCTION

A variety of diphenyl ether(DPE) herbicides such as oxyfluorfen cause rapid peroxidative photobleaching and desiccation of green plant tissues[7,19]. The target site of action of the herbicides is well known to be protoporphyrinogen

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oxidase(Protox), the last common enzyme in the biosynthesis of hemes and chlorophylls[7,15]. Inhibition of Protox by the herbicides leads to excessive accumulation of photosensitizing protoporphyrin IX(Proto) which causes membrane lipid peroxidation in the light[1,7,13,16].

DPE herbicides possess many ideal properties as herbicides ; they are effective at low application dosage and rapidly degraded in environment, and have a broad spectrum of weed control[7,19]. However, only a few plant species such as soybean, peanut, and rice are known to be relatively tolerant to the herbicides[6,11,17]. Such narrow crop selectivity limits their use in cultivated areas of many important crops. If crops can be made resistant to the herbicides, the choice and application times of the herbicides would become more flexible, and the market share of the herbicides could considerably be prolonged and expanded.

The development of transgenic plants resistant to DPE herbicides appears to be possible, since Protox originated from some prokaryotes is poorly inhibited by the herbicides[5,9]. Furthermore, the Protox gene from *Bacillus subtilis* has recently been cloned and expressed in *Escherichia coli*[5]. We have recently introduced the Protox gene of *B. subtilis* into tobacco plants and demonstrated that the transgenic expression of the Protox gene generated resistance to oxyfluorfen[4].

In the present study, the nontransgenic and the transgenic tobacco(*Nicotiana tabacum* cv. Xanthi) plants were compared with respect to responses to DPE herbicide oxyfluorfen and under various environmental conditions.

MATERIALS AND METHODS

Chemicals

Technical-grade herbicide oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-trifluoromethyl)benzene] was generously provided by Rohm and Haas Co.(Springhouse, PA, USA). Restriction enzymes and DNA modifying enzymes were purchased from Promega(Madison, WI, USA) and Sigma Chemical Co.(St. Louis, MO, USA). All other reagents were of the highest quality commercially available.

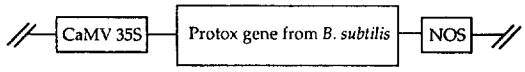

Fig. 1. Structure and chimeric Protox gene construct used for transforming tobacco plants.

Preparation of Protox gene construct and transformation

The Protox gene was isolated from the genomic library of *B. subtilis*(Clontech Laboratories, Inc., Palo Alto, CA, USA) by standard procedures and amplified by polymerase chain reaction (PCR) with primers of 5'-GCCGAAGCTTGGATCCATGAGTGACCGCAAAA-3'(*N*-terminal) and 5'-GCCGAAGCTTGGATCGTTTTAGCTGAATAAAT-3'(*C*-terminal). The 1.4kb PCR product was digested with *Bam*H1 and inserted in the sense orientation between cauliflower mosaic virus (CaMV) 35S promoter and the terminator of nopaline synthase(NOS) gene of the pBI121 vector (Fig. 1). This construct was used to transform tobacco leaf segments via *Agrobacterium* as previously described[4]. The resulting transgenic plants were screened by PCR and a Southern analysis with the Protox gene(1.4kb) as a probe. We expected that the Protox gene under the CaMV 35S promoter would be expressed in the cytosol.

Plant materials

Seeds of the transgenic tobacco plants containing *B. subtilis* Protox gene with CaMV 35S promoter were surface sterilized with 70% ethanol for 30 sec and subsequently 50% Chlorox for 20 min, and then washed thoroughly three times with sterilized distilled water. The seeds were placed

on the Murashige and Skoog medium[18] containing 100mg/l kanamycin. Survival plantlets from the medium were transplanted to Wagner pots(1/5000 a) in a commercial greenhouse substrate and watered with tap water. Following the acclimation of the plants in a growth chamber at 30/20℃, day/night temperature with a 14-h photoperiod for 7 days, they were grown in a greenhouse at 30± 5/20±5℃, day/night temperature with an about 14-h photoperiod until they had reached to a 3- to 4-leaf stage. Seeds of nontransgenic tobacco plants were directly germinated in Wagner pots and grown in the greenhouse in the same manner as employed for the transgenic plants. In separate experiments, some plants were grown under various temperature, light, and water conditions in growth chambers. Growth responses of the transgenic and the nontransgenic plants were determined periodically under the various environmental conditions.

Herbicide treatment

Tobacco tissues from the third and fourth true leaves were treated with various concentrations of oxyfluorfen as before[10,13] by cutting fifty 4-mm diameter leaf discs(approximately 0.2g fresh weight) with a cork borer and then placing them in a 6-cm diameter polystyrene Petri dish containing 5ml of 1% sucrose, 1mM 2-(*N*-morpholino)ethanesulfonic acid(pH6.5) with or without the herbicide dissolved in acetone. Control contained the same amount of the solvent without the herbicide. The final concentration of acetone in all dishes was 1%(v/v). The tissues were incubated in a growth chamber at 25℃ in the dark for 12h and then exposed to continuous white light at 120μmol/m^2/sec photosynthetically active radiation(PAR) for various time periods. No detrimental effects of acetone alone on the tissues were detected during the experiments(data not shown).

Cellular leakage

Cellular leakage was determined periodically by detection of electrolyte leakage into the bathing medium using a conductivity meter(Cole-Parmer Instruments Co., Vernon Hills, IL, USA) as previously described[10]. Because of differences in background conductivity of different treatment solutions, results were expressed as changes in conductivity upon exposure to light.

Lipid peroxidation

Lipid peroxidation was estimated by the level of malondialdehyde(MDA) production using a slight modification of the thiobarbituric acid(TBA) method as previously described[2]. The tissues were treated with oxyfluorfen and incubated in the same manner as used for the measurements of cellular leakage. However, sucrose was omitted from the bathing medium, because it is known to interfere with color development in the assay[2,3,10]. After 24h of exposure to 120μmol/m^2/sec PAR at 25℃, the treated tissues were separated from the bathing medium and keep both fractions in a freezer at -80℃ to avoid further reaction of the tissues with the herbicide until the MDA determination.

The tissues were homogenized with a mortar and pestle in 5ml of a solution of 0.5% TBA in 20% trichloroacetic acid(TCA). The homogenates were centrifuged at 20,000g for 15 min and the supernatants collected. The supernatants were heated in a boiling water bath for 25 min and allowed to cool in an ice bath. Following centrifugation at 20,000g for 15 min, the resulting supernatants were used for spectrophotometric determination of MDA. The aliquots of the bathing medium in which the tissues were incubated with different concentrations of oxyfluorfen were also subjected to the same procedure used for the tissues, using an 1 : 1(v/v) ratio of the aliquot to the solution of 0.5% TBA in 20% TCA. Ab-

sorbance at 532nm for each sample was recorded and corrected for nonspecific turbidity at 600nm. MDA concentration was calculated using a molar extinction coefficient of $156mM^{-1}cm^{-1}$. The MDA concentrations on a fresh weight basis from both fractions of the tissues and the bathing medium were pooled and then regarded as a total MDA produced by the tissues.

RESULTS AND DISCUSSION

Physiological responses of the nontransgenic and the transgenic tobacco plants to DPE herbicide oxyfluorfen were compared with respect to cellular leakage and lipid peroxidation, which have usually been used for assessing the effects of peroxidizing herbicides[1,10,13].

Cellular leakage, as measured by the detection of electrolyte leakage, was determined periodically upon exposure of light at $120\mu mol/m^2/sec$ PAR following 12h incubation in the dark either for the nontransgenic or for the transgenic tobacco leaf discs treated with oxyfluorfen at concentrations from 0.001 to 1,000μM. No significant cellular leakage from both plant tissues was detected at oxyfluorfen concentration of up to 1μM. The cellular leakage from both plant tissues began to increase after 6h light incubation, depending on the concentration of the herbicide and the incubation time. However, the magnitude of the cellular leakage was lower from the transgenic than from the nontransgenic tissues treated with oxyfluorfen(Fig. 2). For example, cellular leakage change from the nontransgenic tissues was approximately 1.4-fold higher than that from the transgenic tissues at 12h after light incubation with 100μM oxyfluorfen.

The level of MDA produced by the leaf discs was determined as an estimate of lipid peroxidation following the oxyfluorfen treatment. Since MDA is water-soluble and is a relatively small

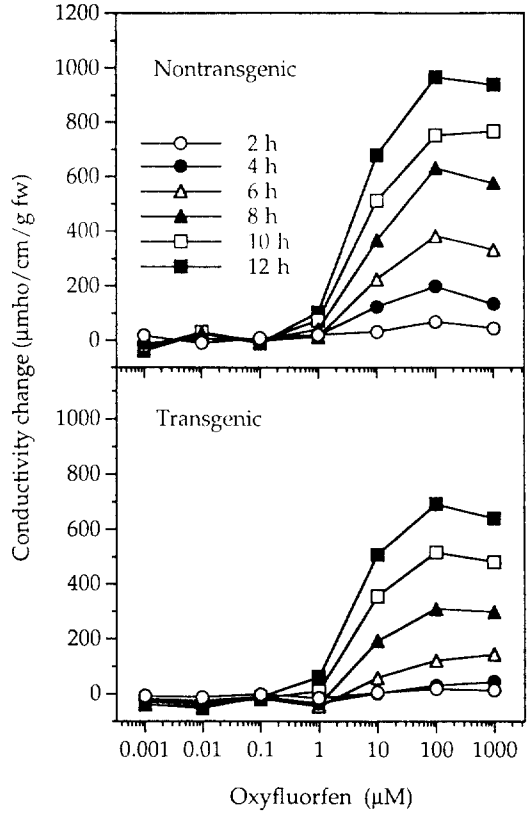

Fig. 2. Effect of oxyfluofen on electrolyte leakage from leaf discs of the nontransgenic and the transgenic tobacco plants. The tissues were exposed to continuous light at $120\mu mol/m^2/sec$ at 25℃ following 12h dark incubation. Values are differences between treated and control tissues.

molecule with two aldehyde groups, it will be leached out from the leaf discs into the incubation media if the plasma membrane(PM) is damaged or broken[3,10]. Thus, we measured the MDA concentration from the incubation media as well as from the leaf discs. Oxyfluorfen at a concentration of higher than 0.1μM caused considerable lipid peroxidation in the nontransgenic tissues, but lesser degree of lipid peroxidation was detected in the transgenic tissues(Fig. 3). However, no further lipid peroxidation either in the nontransgenic or in the transgenic tissues occurred beyond 10μM oxyfluorfen concentrations. When compared to the

cellular leakage caused by oxyfluorfen(Fig. 2), appreciable lipid peroxidation could occur with about one order less oxyfluorfen concentration (Fig. 3). These results suggest that cellular leakage caused by oxyfluorfen is due to the consequence of lipid peroxidation on PM and other membranes, confirming the previous findings[7,19].

The observations that both cellular leakage and lipid peroxidation caused by oxyfluorfen were less in the transgenic than in the nontransgenic plants (Figs. 2 and 3) led us to determine whether the transgenic plants exhibit normal growth pattern. Growth responses of the nontransgenic and the transgenic plants were compared under various environmental conditions. Both plants were subjected to the environmental conditions following the acclimation at 30/20℃, day/night temperature with a 14-h photoperiod for 7 days.

When the plants were subjected to minimum threshold temperature(15℃), optimum temperature (25℃), or maximum threshold temperature(35℃) condition for their growth, the nontransgenic and the transgenic plants exhibited similar growth response, in terms of plant height, to each temperature condition(Fig. 4). Both plants were also grown at an almost the same rate under light condition of 100 or 200μmol/m^2/sec PAR(Fig. 5). At 20μmol/m^2/sec PAR, however, the transgenic plants exhibited slightly more retarded growth

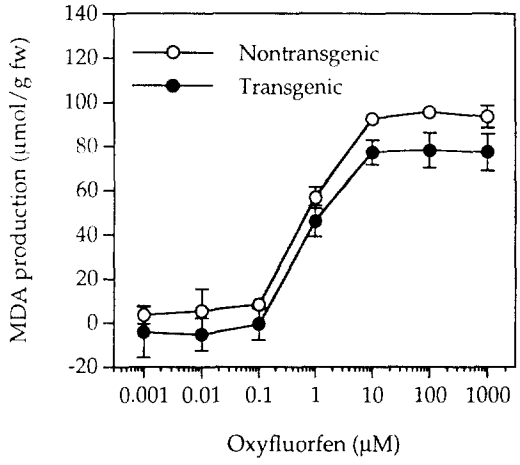

Fig. 3. Effect of oxyfluofen on MDA production from leaf discs of the nontransgenic and the transgenic tobacco plants. The tissues were exposed to continuous light at 120μmol/m^2/sec at 25℃ following 12h dark incubation. Error bars are ±1 standard error of the means. In some cases the error bar is obscured by the symbol.

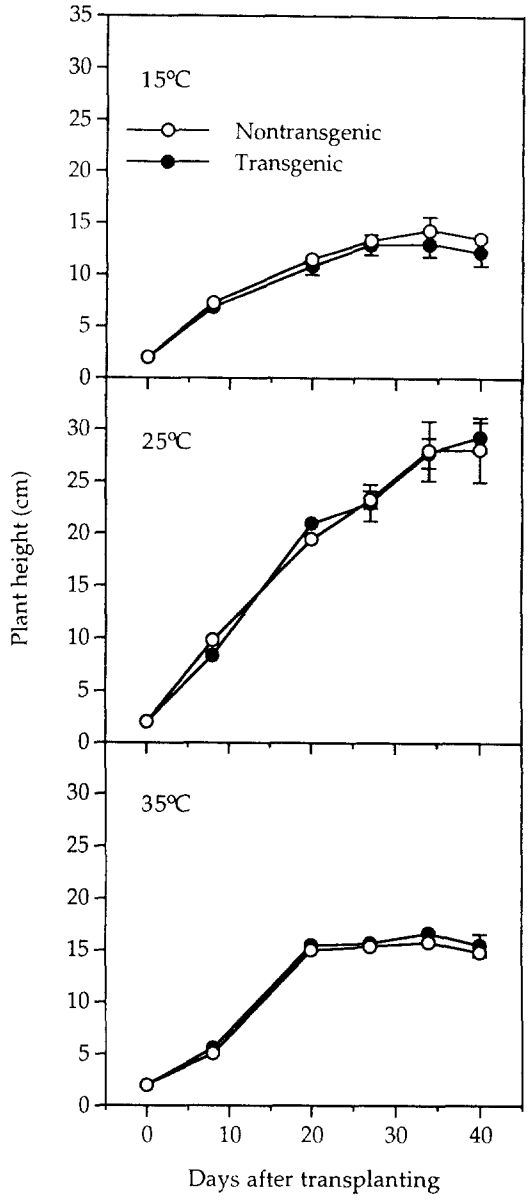

Fig. 4. Growth of the nontransgenic and the transgenic tobacco plants under various temperature conditions.

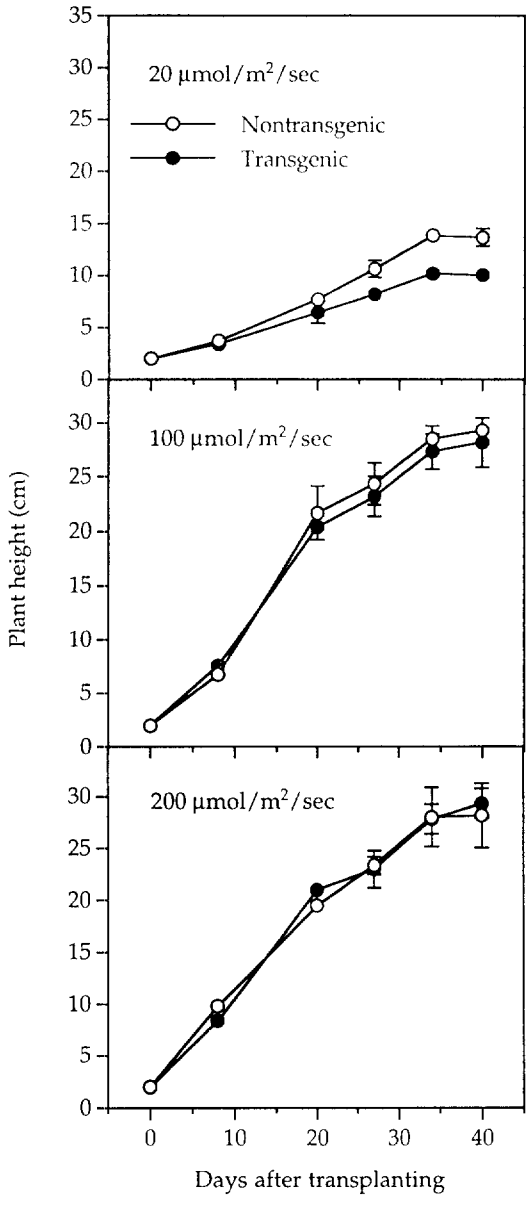

Fig. 5. Growth of the nontransgenic and the transgenic tobacco plants under various light conditions.

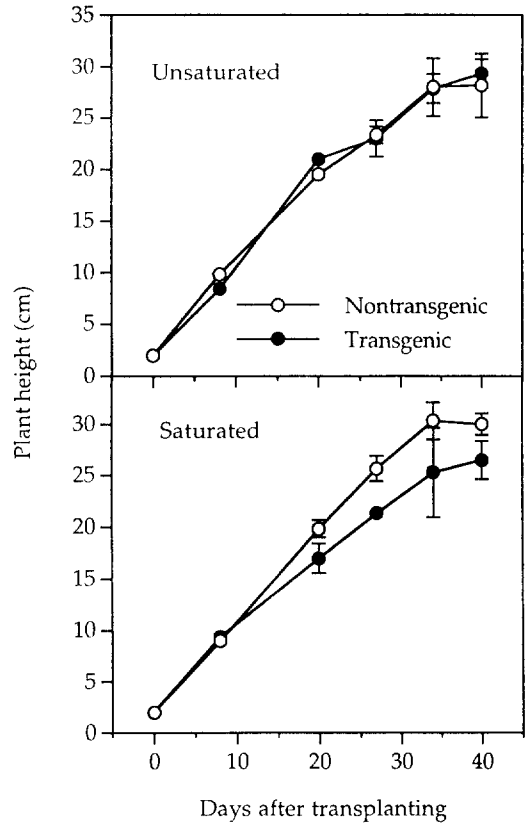

Fig. 6. Growth of the nontransgenic and the transgenic tobacco plants under unsaturated and saturated water conditions.

than the nontransgenic plants(Fig. 5). The growth pattern of the nontransgenic plants was not different under unsaturated and saturated water conditions during the experiment(Fig. 6). However, the transgenic plants under saturated water condition exhibited slightly lower growth rate than those under unsaturated water condition, and than the nontransgenic plants under unsaturated or saturated water condition(Fig. 6). Despite the slightly retarded growth of the transgenic plants under the adverse growth conditions, the growth responses of the transgenic plants under various temperature, light, and water conditions were generally similar to those of the nontransgenic plants. In addition, other growth responses such as shoot fresh weight and number of leaves were similar in the transgenic and in the nontransgenic plants under the various environmental conditions (data not shown).

In summary, both cellular leakage and lipid peroxidation caused by oxyfluorfen were found to be less in the transgenic than in the nontransgenic tobacco plants. Growth responses of the

transgenic plants under various temperature, light, and water conditions were almost the same as those of the nontransgenic plants, except the growth of the transgenic plants under low light or saturated water condition. These results imply that the growth of the transgenic plants has not been altered due to the gene transformation. Thus, it can be concluded that the transgenic tobacco plants containing *B. subtilis* Protox gene with CaMV 35S promoter are relatively resistant to oxyfluorfen and grow normally.

DPE herbicides including oxyfluorfen have been reported to effectively inhibit Protox both in the plastid and in the mitochondrion[7,9,15]. Protoporphyrinogen IX(Protogen), the substrate of Protox, is readily exported from the herbicide-inhibited plastid[8], since it is not lipophilic[14]. Protogen is oxidized to Proto by the herbicide-insensitive peroxidase-like activity in the pM[12,14]. Abnormally high levels of lipophilic Proto accumulate in the PM and other membranes, and the accumulated Proto causes photodynamic membrane lipid peroxidation and ultimate cellular death[12,14]. In the transgenic plants, however, Protogen could be oxidized to Proto by the herbicide-insensitive *B. subtilis* Protox expressed in the cytosol before it is transproted to the PM. Because of high lipophilicity of Proto[14], it could be transported from the cytosol to the plastid and the mitochondrion[7,20], and then be utilized as a substrate for Mg- and Fe-Proto chelatase, respectively. Proto could also be partitioned into the PM, which is devoid of chelatase activity, but the accumulation of Proto could much be alleviated. This fact might account for the resistance of the transgenic plants containing *B. subtilis* Protox with CaMV 35S promoter to DPE herbicides. However, this proposed resistance mechanism should further be substantiated in the near future.

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적 요

Bacillus subtilis protoporphyrinogen oxidase 유전자를 *Agrobacterium*을 매개체로 이용하여 전이시킨 후 cauliflower mosaic virus 35S promoter하에서 발현케 한 형질전환 담배를 유기하였다. 이러한 형질전환 담배의 diphenyl ether계 제초제 oxfluorfen에 대한 생리적 반응과 여러 환경 조건에서의 생장 반응을 재배종 담배와 비교하였다. Oxyfluorfen의 처리에 의해 나타나는 세포내 구성물질의 누출과 지질과산화작용은 재배종 담배에서보다 형질전환 담배에서 더 작게 이루어졌다. 형질전환 담배의 생장을 여러 온도, 광도 및 수분 조건에서 조사한 결과, 저광도와 포화수분 조건에서의 생장이 재배종 담배에 비해 다소 저하되는 현상이 나타났을 뿐 기타 조건에서의 생장은 재배종 담배의 생장과 거의 동일하였다. 따라서 *B. subtilis* protoporphyrinogen oxidase 유전자를 전이시켜 cauliflower mosaic virus 35S promoter하에서 발현케 한 형질전환 담배는 oxyfluorfen에 대해 비교적 높은 저항성을 나타내지만 형질전환에 따른 생장 변화는 크게 일어나지 않음을 알 수 있었다. 한편 이러한 형질전환 담배의 oxyfluorfen에 대한 저항성 기작에 대해서도 논의하였다.

REFERENCES

1. Becerril, J.M. and S.O. Duke. 1989. Protoporphyrin IX content correlates with activity of photobleaching herbicides. Plant Physiol. 90 : 1175-1181.
2. Buege, T.A. and S.D. Aust. 1978. Microsomal lipid peroxidation. Methods Enzymol.

52 : 302-310.

3. Choi, G.J., H.J. Lee and K.Y. Cho. 1996. Lipid peroxidation and membrane disruption by vinclozolin in dicarboximide-susceptible and -resistant isolates of *Botrytis cinerea*. Pestic. Biochem. Physiol. 55 : 29-39.
4. Choi, K.W., O. Han, H.J. Lee, Y.C. Yun, Y. H. Moon, M. Kim, Y.I. Kuk, S.U. Han and J.O. Guh. 1998. Generation of resistance to the diphenyl ether herbicide, oxyfluorfen, via expression of the *Bacillus subtilis* protoporphyrinogen oxidase gene in transgenic tobacco plants. Biosci. Biotechnol. Biochem. 62 : 558-560.
5. Dailey, T.A., P. Meisner and H.A. Dailey. 1994. Expression of a cloned protoporphyrinogen oxidase. J. Biol. Chem. 269 : 813-815.
6. Duke, S.O., H.J. Lee, M.V. Duke, K.N. Reddy, T.D. Sherman, J.M. Becerril, U.B. Nandihalli, H. Matsumoto, N.J. Jacobs and J. M. Jacobs. 1997. Mechanisms of resistance to protoporphyrinogen oxidase-inhibiting herbicides. *In*. R. De Prado, J. Jorrin and L. Garcia-Torres(eds.). Weed and Crop Resistance to Herbicides. Kluwer Academic Publishers, Dordrecht, pp. 155-160.
7. Duke, S.O., J. Lydon, J.M. Becerril, T.D. Sherman, L.P. Lehnen and H. Matsumoto. 1991. Protoporphyrinogen oxidase-inhibiting herbicides. Weed Sci. 39 : 465-473.
8. Jacobs, J.M. and N.J. Jacobs. 1993. Porphyrin accumulation and export by isolated barley(*Hordeum vulgare* L.) plastids : Effect of diphenyl ether herbicides. Plant Physiol. 101 : 1181-1187.
9. Jacobs, J.M., N.J. Jacobs, S.F. Borotz and M.L. Guerinot. 1990. Effects of photobleaching herbicide, acifluorfen-methyl, on protoporphyrinogen oxidation in barley organelles, soybean root mitochondria, soybean root nodules, and bacteria. Arch. Biochem. Biophys. 280 : 369-375.
10. Kenyon, W.H., S.O. Duke and K.C. Vaughn. 1985. Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs. Pestic. Biochem. Physiol. 24 : 240-250.
11. Komives, T. and G. Gullner. 1994. Mechanism of plant tolerance to photodynamic herbicides. Amer. Chem. Soc. Symp. Ser. 559 : 177-190.
12. Lee, H.J. and S.O. Duke. 1994. Protoporphyrinogen Ⅸ-oxidizing activities involved in the mode of action of peroxidizing herbicides. J. Agric. Food Chem. 42 : 2610-2618.
13. Lee, H.J., M.V. Duke, J.H. Birk, M. Yamamoto and S.O. Duke. 1995. Biochemical and physiological effects of benzheterocycles and related compounds. J. Agric. Food Chem. 43 : 2722-2727.
14. Lee, H.J., M.V. Duke and S.O. Duke. 1993. Cellular localization of protoporphyrinogen-oxidizing activities of etiolated barley(*Hordeum vulgare* L.) leaves : Relationship to mechanism of action of protoporphyrinogen oxidase-inhibiting herbicides. Plant Physiol. 102 : 881-889.
15. Matringe, M., J.M. Camadro, P. Labbe and R. Scalla. 1989. Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. Biochem. J. 260 : 231-235.
16. Matringe, M. and R. Scalla. 1988. Effects of acifluorfen-methyl on cucumber cotyledons : Protoporphyrin accumulation. Pestic. Biochem. Physiol. 32 : 164-172.
17. Matsumoto, H., J.J. Lee and K. Ishizuka. 1994. Variation in crop response to protoporphyrinogen oxidase inhibitors. Amer. Chem. Soc. Symp. Ser. 559 : 120-132.
18. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 :

473-497.

19. Scalla, R. and M. Matringe. 1994. Inhibitors of protoporphyrinogen oxidase as herbicides : Diphenyl ethers and related photobleaching molecules. Rev. Weed Sci. 6 : 103-132.

20. Smith, A.G., O. Marsh and G.H. Helder. 1993. Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants. Biochem. J. 292 : 503-508.