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Immunological Detection of Cytosolic Immature and Plastidial Mature EPSP-synthase after Glyphosate Treatment in Tomato(*Lycopersicon esculentum*) Apical Meristem

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Glyphosate 처리후 토마토 정단부 세포질과 원형 엽록체에서 immature 및 mature EPSP-synthase의 면역학적 검정

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ABSTRACT

Glyphosate had no effect on 5-enolpyruvylshikimate-3-phosphate synthase(EPSP-synthase) biosynthesis *per se*. But it inhibited clealy the activity of EPSP-synthase. EPSP-synthase seemed to be synthesized as a higher molecular weight(54 kDa) presusor protein and to be transported into plastid. The apparent molecular weight of mature EPSP-synthase in plastid is 45 kDa. Thus, the molecular size of transit peptide appeared to be about 9 kDa. The etiolation for 48 h after glyphosate application did not exhibit the inhibition of translocating level of EPSP-synthase across chloroplast envelope in actively growing meristematic leaves. But even when the plants were etiolated 2 hr after glyphosate treatment, a complete inhibition did not occur at least within 12 hr, *i.e.* 2 hr after beginning light period, suggesting that EPSP-synthase biosynthesis appeared to be not completely light dependent and the level of EPSP-synthase translocation to chloroplast could be controlled by an unknown regulatory mechanism of light dependent herbicidal effect of glyphosate.

Key words : glyphosate, immunological detection

INTRODUCTION

Glyphosate is a potent herbicide which inhibits EPSP-synthase[15,28]. Although EPSP-synthase of the shikimic acid pathway is the herbicidal target enzyme[19,20], it is not clearly understood whether the inhibition effect of glyphosate is on EPSP-

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synthesis in transcriptional level or only on the enzymatic activity. During chloroplast swelling in the glyphosate-treated meristem of tomato plants, it might be necessary to induce a change of translocation of nuclear encoded EPSP-synthase into chloroplast.

Many chloroplast proteins are encoded by nuclear DNA, synthesized on cytosolic ribosomes as precursors with transient N-terminal extension called a transit peptide or with specific C-terminal sequence, and imported across the envelope membranes to reach the stroma[6,18,24,26,30]. The transit peptide of precursor proteins which are destined to be inserted to thylakoids is cleaved off by certain protease present in the lumenal side of the thylakoid membrane[23].

Little is known a subcellular redistribution of EPSP-synthase after translation on cytosolic ribosomes[14]. In present work, it is reported that EPSP -synthase is translocated into stromal side after a cleavage of transit peptide with relatively large molecular weight(*ca* 9 kDa).

MATERIALS AND METHODS

Plant materials and growth condition

The apical meistem and leaves of six week-old tomato, *Lycopersicon esculentum* Mil vat. Moneymaker, was used for the measurement of EPSP-synthase activity. The isolation of cytosolic or plastidial proteins was preformed with same parts. The treatment of glyphosate and growth condition were followed as previous report by Kim and Amrhein[19]. All parts are excised with a razer blade and reserved under N_2 gas.

Chloroplast isolation

Chloroplasts and thylakoids were isolated from fresh apical meristem. Chloroplast isolation was carried out as previous method by Kim and Heinrich[21,22], except for twice washing step in homogenizing buffer, 50mM Tricine-NaOH(pH 7.8), 400mM sucrose, 5mM $MgCl_2$, and 10mM NaCl. Chloroplast proteins were subjected to SDS -PAGE after hypotonical rupture in same buffer without sucrose.

Cytosolic protein isolation

The tomato apical meristems were chopped with razor blade in an extraction buffer(1 : 2, w/v) consisted of 25mM Mops-Tris, pH .7.5, 5 mM $MgCl_2$, 10% sucrose, 0.6% PVP, 5mM EDTA and 0.2mM EGTA, 1mM PMSF and 20 mM β-mercaptoethanol. The leaves were ground with mortar and pestle. After the filtration through 8 layers of cheese chlothes, the filtrates were pelleted by $1,000 \times g$ centrigugation for 15min at 2C to remove cell debris. The supernatants were recentrifuged at $6,000 \times g$. The supernatants were repelleted through $150,000 \times g$ with Beckman rotor 27.1. The supernatants and pellets were used as soluble. The microsomal proteins were twice washed in a washing medium consisted of 25mM Mops-Tris(pH 7.5), 10% sucrose, 1mM PMSF, 10mM β-mercaptoethanol and 0.2% Triton X-100, and then stored at -80C until analysis. The supernatants were dialysed against the washing medium without Triton X-100 overnight. All precedures were performed by $21 \pm$C. Protein contents were measured by Bradfords method[3].

EPSP-synthase activity

Enzyme activity was spectrophotometrically measured with cytosolic proteins as previously reported by Kim and Amrhein[20] using Lanzetta's phosphate dye binding method[16].

Immunodetection of EPSP-syntahse

After SDS-PAGE[17], the proteins were electrophoretically transferred to nitrocellulose sheet as Towbin et al's method[32]. Immunoblotting was carried out as previous method by Kim and

Amrhein[21] exception for the use of monoclonal EPSP-synthase antibody.

RESULT AND DISCUSSION

Figure 1 shows the changes in EPSP-synthase activities in plant parts 3 days after 100 nmole glyphosate treatment. According to source to sink theory, the *in vivo* inhibiton of EPSP-synthase activity is not obiously in the old leaves(leaf 1 to 5) exception for leaf 3 on which glyphosate is loaded. In real the assimilate exporting old leaves(source) does not show an accumulation of shikimic acid through the inhibition of EPSP-synthase[19]. But the apical meristems from root and stem including leaf primordia and young growing leaf(< 1cm long) are strongly affected by glyphosate. As shown in Fig. 2, the inhibition of EPSP-synthase activity in apical meristem from stem is induced within 16 h after glyphosate application. EPSP-synthase activity seems to be changed with growth development. The younger leaves have higher activities, meaning that actively developing leaves need to synthesize more EPSP-synthase. Arnaud *et al.*[2] suggested that high

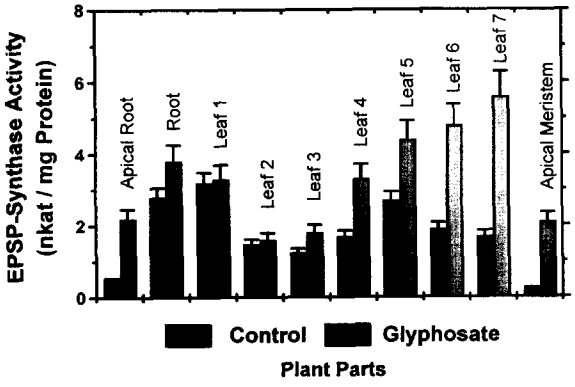

Fig. 1. EPSP-synthase activities in each plant part. Plants were sampled 3 days after treatment with 200 nmole glyphosate. Vertical bars mean the standard errors(SE) of three independent experiments. Vertical bars(SE) are missing where the SE was smaller than symbol size.

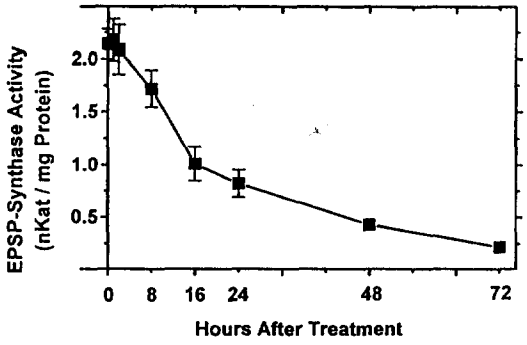

Fig. 2. Chronological EPSP-synthase activities in apical meristems for 72 hr after treatment with 200 nmole glyphosate.Vertical bars mean the standard errors(SE) of three independent experiments. Vertical bars(SE) are missing where the SE was smaller than symbol size.

enzyme activity in the wheat coleoptile for the whole life time might be associated with its lignification. Leaf expansion process is necessary to accompany organogenesis like as xylem- and phloem-development *i.e.* vasicularization. During the vasicularization, lignin biosynthesis is more actively induced. Shikimic acid pathway plays a key role in aromatic amino acids and lignin biosynthesis. The EPSP-synthase activity in apical meristem seems to have about 4 to 6 nkat per mg protein compared to 2 nkat per mg protein in older leaves(leaf 1 to leaf 3). Lower activity in older leaves(leaf 1 and 2) than in younger leaves(leaf 4 to 7) is thought to be due to leaf senescence following chlorosis[19]. EPSP-synthase acivity in the root meristem also reduced.

Figure 3 shows the immunoblotting analysis of EPSP-synthase. The apparent molecular weights of precursor and mature EPSP-synthase 54kDa and 45kDa are smaller than 64.5kDa and 59kDa in plastid-free *Euglena* mutant as reported by Reinbothe *et al*[25]. Interestingly, 54kDa precursor form appears to be enzymatically active(unpublished data). The apparent molercular size of transit peptide(about 9kDa) is very similar to one of ribulose bisphosphate carboxylase small subu-

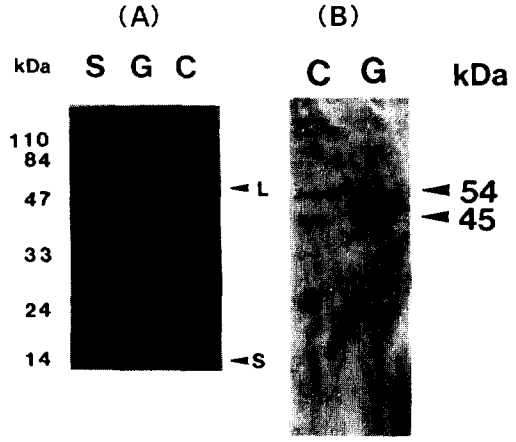

Fig. 3. Seperation(A) on a 12.5% SDS-PAGE of cytosolic soluble protein(25 μg protein) from tomato apical meristem. Glypgosate was applicated 2 hr before dark period. The proteins were extracted 12 hr after glyphosate treatment, *i.e.* 2 hr after biginning light period(16/8 hr light/dark). After the polypeptide were electrotransfered onto a nitrocellulose membrane, the immunoblotting was carried out using the antibody raised against the EPSP-synthase at room temperature. Antibody complexes conjugated with alkaine phosphatase were visualized using 5-bromo-4chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates(B). +G, 200 nmole glyphosate treatment on the middle lobe of the third old leaf ; -G, nontreated control.

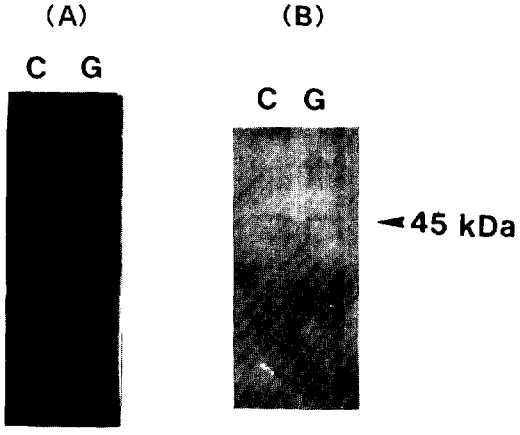

Fig. 4. Chloroplasts from apical meristems were isolated. After hypotonical rupture, proteins(25 μg chlorophyll) were seperated(A) on a 12.5 % SDS-PAGE. After the polypeptide were electrotransfered onto a nitrocellulose membrane, the immunoblotting was carried out using the antibody raised against the EPSP-synthase at room temperature. EPSP-synthase was visualized using its antibody and secondary antibody conjugated with alkaine phosphatase(B). +G, 100 nmole glyphosate treatment on the middle lobe of the third old leaf ; -G, nontreated control ; S, molecular weight standard.

nit[6,13], but more than twice as large as the transit peptide of the light harvesting chlorophyll a/b binding protein[27,29,30]. Chloroplasts are surrounded by double-membrane envelopes. One of the specific features of posttranslational protein translocation mechanism across plastidial and mitochondrial membranes is that proteins have to be at least partially unfolded upon passage through their destined membranes[4,5,7,8,33]. For *Euglena* mutant, the precursor EPSP-synthase anchored to plastid must lose a direction for organellar redistribution. Thus it is difficult to deduce a conclusion why the size difference in transit peptide and mature EPSP-eynthase between higher plant and *Euglena* occurs. In present work, it is confirmed that EPSP-synthase activity is inhibited through glyphosate. However, it is needed to identify whether the inhibition is limited to cytosolic precursor protein or plastidial mature protein. Further understanding of the mode of action of glyphosate must wait for the determination of EPSP-synthase substrate levels in plastids and in the cytoplasm. There are some evidences which suggested protein translocation could be inhibited by biological toxins and inhibitors. For exemple, the presence of methotrexate inhibited the import of dihydrofolate reductase into mitochondria[1]. Similarily, the translocation of EPSP-synthase into chloroplast could be deterred by glyphosate. Della-Cioppa and Kishore[7] reported that glyphosate *per se* inhibits the import of plastidial precusor proteins. However, in present work, the inhibition is incomplete(Fig. 3), *i.e.* the EPSP-synthase bands

on immunoblot does not clearly disappear. It might suggest that the detected EPSP-synthase had been translocated to chloroplast before glyphosate treatment. It implies that glyphosate translocation is somewhat dependent on light. It should be noted that glyphosate was applied 2 hr before dark period(Fig. 4) and sampled 2 hr after beginning light period. Sampling time might be so short that the light dependent herbicidal effect of glyphosate could exert toxicity.

Thus the etiolation test was carried out to illuminate whether the translocation of EPSP-synthase is light dependent or not. Fig. 5 shows the immunoblottings of EPSP-synthase in etiolated apical meristem for 48 hr. An immediate etiolation(EI) after glyphosate application does not completely exert an inhibitory effect on the intraorganellar distribution of EPSP-synthase. This means paradoxically that the inhibition of translocation is induced only after glyphosate is assimilated in apical meristems under illumination and then activated. As seen in lane EA of Fig. 5, the immuno-blotted band of EPSP-synthase within chloroplast are weakly detected, indicating that light has an effect no matter what it is direct or not. We would assume that light is not essential factor since a relationship between light signaling and plastidial response induced by glyphosate activation is not clealy illucidated. There may be some reasonable interpretations as following ; the changes in envelope lipid comopsition and membrane electrochemical potential($\Delta\Psi$) during the chloroplast swelling induced by glyphosate could explain the inhibition mechanism. But since chloroplastic protein import requires ATP alone[26], it does not appear that a membrane potential difference by excess shikimic acid accumulation is a cause of the inhibition. It is noted that mitochondrial protein import requires both ATP and a potential across the inner membrane[8].

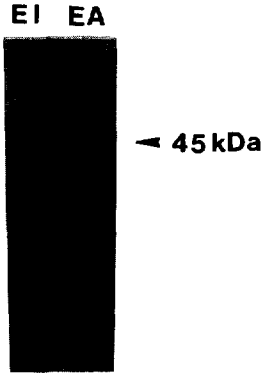

Fig. 5. Proteins were extracted from the chloroplasts of apical meristems after etiolation. After hypotonical rupture of chloroplast and twice washing step, proteins(25μg chlorophyll) were seperated on a 12.5% SDS-PAGE. After the polypeptide were electrotransfered onto a nitrocellulose membrane, the immunoblotting was carried out using the antibody raised against the EPSP-synthase at room temperature. EPSP-synthase was visualized using its antibody and secondary antibody conjugated with alkaine phosphatase. EI, 48 hr-etiolation immediately after 200 nmole glyphosate treatment ; EA, 48 hr-etiolation 48 hr after 200 nmole glyphosate treatment.

On the other hand, in previous work it has also been well known that the decrease in turn over rate of QB protein within LHC II is included in a pleiotropic toxicity of glyphosate[21]. The decrease in turn over rate of QB protein reduces electron transfer and thereafter ATP-biosynthesis. Furthermore, recent finding indicates that two EPSP-synthase isoforms are also localized in plastid and the biosynthesis rate of both isozymes is different during cell proliferation[10]. However, nothing is known about the conformational change of a precursor protein and the isozymes during translocation across chloroplast envelope and about the protein-unfolding activity[9]. In future work, it needs to illuminate the disorder of the translocation mechanism across the inner and outer envelope membrane associated with a pH dissipation within chloroplasts and/or thylakoidal lumens under a chloroplast swelling status and

ATP-depletion by glyphosate.

Glyphosate is symported with sucrose through phloem[28]. Although it is well known that many nuclear encoded proteins are light dependent and regulated through phytochrome system[11,31], it could be concluded that light is not necessary to induce the expression of EPSP-synthase, suggesting that there is more complex relationship among some factors regulating chloroplast vitality like as sucrose symport to meristem, ATP synthesis and light signaling.

Our next report will be a change in EPSP-synthase activities within chloroplast and of cytosolic premature EPSP-synthase to identify how glyphosate does exhibit a different inhibition mechanism between premature 54kDa and mature 45kDa.

摘　要

除草除 glyphosate는 EPSP-synthase 生合成 자체는 抑除하지 못하며 단지 酵素活成만을 억제한다. 이러한 억제는 뿌리 및 줄기 정단부에서 현저했다. EPSP-synthase의 前體驅 蛋百質은 54 kDa의 分子量을 갖으며, 葉綠體내 成熟 蛋百質은 45kDa 정도의 分子量을 나타낸다. Glyphosate 처리 直後 인위적 暗處理는 EPSP-synthase의 葉綠體내 삽입을 완전히 억제하지 못한다. 이러한 결과는 Glyphosate에 의한 EPSP-synthase 葉綠體내 삽입 기작은 전적으로 光의존적인 것은 아니며 오히려 광의존적 제초활성을 나타내는 glyphosate의 엽록체내 유입후 아직까지 알려지지 않은 조절기작에 의해 간접적으로 억제되는 것으로 보였다.

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