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Phenotypic Alterations in Transgenic Tobacco Plants that Overproduce Cytokinins

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Cytokinins overproduction에 따른 담배형질전환체의 변화

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Cytokinin is one of major growth regulators in plants. In this study, the gene isopentenyl transferase (*ipt*) which encodes a key enzyme involved in the biosynthesis of the growth regulator cytokinin isolated from *Agrobacterium tumefaciens* was introduced into tobacco plant via *Agrobacterium*-mediated transformation. The *ipt* gene was modulated using the proteinase inhibitor II (PI-IIK) promotor. In general, this promoter/*ipt* gene fusion resulted in overproduction of cytokinins throughout the transgenic plants. The overproduction of cytokinin caused dramatic changes in morphology of the plant, including stem thickness and reduced root development. The studies reported in this paper were initiated to examine the consequences of overproduction of cytokinin in plant.

식물의 주요 phytohormone**의 하나인** cytokinin**은 식물체의 줄기와 뿌리성장 그 외에도 영양의 전달이나 노화방지, 열매숙성등 식물의 성장과 발달에 미치는 영향은 크고 다양하다.** Cytokinin **생합성에 관여하는 효소를 생산하는 것으로 알려져 있으며 토양박테리아** *Agrobacterium tumefaciens***에 존재하는 유전자인** isopentenyl transferase (*ipt*)**를 이용한 많은 분자생물학적 연구가 진행되어 왔는데 그 중 하나로 이 연구에서** *ipt* **유전자에 의한** cytokinin**의** overproduction**이 식물체에 성장과 발달에 어떠한 영향을 주는지 관찰하고 그 결과가 제시할 수 있는 작물의 유전 공학적 이용가능성에 대하여 알아본다.**

Key words : Cytokinin, *Agrobacterium tumefaciens*, isopentenyl transferase (*ipt*) gene, proteinase inhibitor II promoter, transformation.

I. Introduction

Plant growth and development are affected by phytohormones in many aspects. Cytokinins are a class of phytohormones defined as substances that stimulate cell division in plants and that interact with auxin in determining the direction which differentiation of cell takes (Wareing and Philips, 1970). Cytokinins are

thought to control many growth and developmental processes in plants, such as shoot and root formation, apical dominance, source-sink relation, senescence, cell differentiation, and cell extension (Delvin and Witham, 1983; Horgan, 1984). Externally applied cytokinins exhibit a wide variety of effects, including shoot initiation from callus cultures, promotion of axillary bud growth, directed transport of nutrients, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence. Cytokinins also have been implicated in the physiological and biochemical process with marked effects on flowering, fruit set and ripening, leaf senescence, seed germination, and stamatal function.

While the traditional approaches with the external application of cytokinins have provided a large amount of information on the hormonal action of cytokinins, they are limited by a general lack of understanding of the mechanisms of cytokinin uptake, transport, and action at cellular sites. In an effort to circumvent some of the problems associated with the traditional approaches, recent molecular approaches involving gene transfer technology has been taken to determine the role of cytokinin in plants (Smigocki *et al*., 1993; Medford, *et al*., 1989; Martineau *et al*., 1995; Li *et al*., 1992). Guilfoyle and colleagues transformed cytokinin biosynthetic gene (*ipt*) from *Agrobacterium tumefaciens* into tobacco plant and studied the roles of cytokinin in the transgenic plant (Li *et al*., 1992). They have found that an overexpression of bacterial isopentenyl transferase (*ipt*) gene in the transgenic tobacco plant altered plant morphology, including stunting, loss of apical dominance, reduction in root initiation and growth, either acceleration or prolonged delayed senescence in leaves depending on the growth conditions, adventitious shoot formation from unwounded leaf veins and petioles, altered nutrient distribution, and abnormal tissue development in stems. The result indicated that cytokinin affects plant growth and development in various aspects. In addition, others showed that controlling cytokinin level in plant may give useful traits in biotechnological aspects (Smigocki *et al*., 1993; Martineau *et al*., 1995); When the bacterial isopentenyl transferase (*ipt*) gene was fused with a promoter from the proteinase inhibitor II gene and introduced into the tobacco plant, the transgenic plant displayed enhanced insect resistance, suggesting that the cytokinin gene product mediates defensive properties against insects (Smigocki *et al*., 1993). Also, Martineau et al. (1995) suggests that expression of *ipt* gene in tomato ovaries causes increases in solids content of tomato fruit. Taken together, these results suggest that the manipulation of cytokinin level gives influences on plant development depending on the growth conditions.

In order to understand its role in other plant species, *Nicotiana tabaccum* has been chosen. The transgenic plant undergone an overproduction of cytokinin showed significant morphological changes on the vegetative growth and development. The detailed experimental studies are discussed.

II. Materials and Methods

1. Bacterial strains, plant materials, and plant transformation

Escherichia coli MC1000 (*ara, leu, lac, gal, str*) was used as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 (Hoekema *et al*., 1983) containing the Ach5 chromosomal background and a disarmed helper-Ti plasmid pAL4404, was used for transformation of tobacco plants (*Nicotiana tabaccum L. cv. Xanthi*) by the cocultivation method (An *et al*., 1988). Transgenic plants were maintained in greenhouse conditions.

2. Chimeric construction between *ipt* gene and PI-IIK promoter.

In order to clone the *ipt* gene directly from T-DNA region of Ti-plasmid, T-DNA was digested with *Hin*dIII and *Kpn*I restriction

enzymes that release approximately 1.08 kb of DNA fragment containing the *ipt* gene, and the DNA fragment was subcloned into the same re striction site of *pBluescript* vector (Staratagene). Two oligo-primers, IPT5 (CCTCGAGCCATGGA CCTGCATCTAAT) that recognizes 5' end of the *ipt* gene and T7 (GTAATACGACTCACTA TAGGGC) that anneals the multiple cloning region of theplasmid vector (right beyond the *Kpn*I cloning site), was used to amplify the *ipt* gene (Fig. 1).

Fig. 1. Construction of the chimeric transgene between PI-IIK promoter and ipt gene.
Two oligo-primers, IPT5 and T7, was used to amplify ipt gene from the T-region of Ti-plasmid. The isolated ipt gene transcript was placed under the PI-IIK promoter. LB; left border, RB; right border in the binary vector, pGA628.

The amplified *ipt* gene was sequenced in part and was partially replaced with the original DNA fragment to avoid possible PCR error. The *ipt* gene was then placed under the proteinase inhibitor II (PI-IIK) promoter.

3. Northern blot analysis

Total RNA was isolated from the transgenic tobacco leaves by the guanidium thiocyanate-CsCl method. Twenty-five μg RNA was separated by running an 1.3% agarose gel in MOPS buffer (0.02 M of 3-N-morpholinopropanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, and 2% formaldehyde). The separated RNA was then transferred onto a nylon membrane, and was hybridized with radioactively labeled probe at 60C in the solution containing 0.5 M NaPO4 (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS (Church, 1984).

III. Results and Discussions

Cytokinin is known to regulate normal growth and development of plant. Relative high cytokinin level in tissue culture causes shoot formation, but suppresses root formation, whereas opposite effects are caused by relative high level of auxin (Skoog and Miller, 1957). The discovery that *Agrobacterium* contains the *ipt* gene which encodes a key enzyme involved in cytokinin biosynthesis brought ideas to study roles of cytokinin in plants. Recent molecular studies involving *ipt* gene transfer into plants showed various morphological changes in vegetative organs (Li *et al*., 1992; Chaudhury *et al*., 1993). Not only for the influence on vegetative growth of plant by cytokinin, but also it has been reported that cytokinin influences on defense mechanism of plant (Memelink *et al*., 1987); increase of cytokinin production in plant induces plant defense-related mRNA, encoding extensin, chitinase, PR-1 and PR-1-like

proteins. Previously, the PI-IIK promoter has been used to express the *ipt* gene in tobacco plant, *Nicotiana plumbaginifolia* (Smigocki *et al.*, 1993). In the study, cytokinin level was elevated by about 70-fold by the promoter in the transgenic plant. Surprisingly, the increase of cytokinin production enhanced insect resistance, suggesting that cytokinin may also involve secondary metabolic pathway to induce plant resistant genes.

In order to study influences of cytokinin overproduction on other plant species, *Nicotiana tabaccum* was chosen, and the *ipt* gene driven by PI-IIK promoter was introduced into the plant via *Agrobacterium*-mediated transformation method. A total of 20 independent transgenic plants was regenerated and studied. To examine the transgene (*ipt*) expression in the transgenic plant, northern blot analysis was conducted with 25 μg of total RNA isolated from the leaves of the transgenic plants and few showed strong expression of the *ipt* transcript (data not shown). Of the plants showed the strong expression, few plants exhibited altered morphol-ogy in growth including stem thickening and reduced root development (Fig. 2).

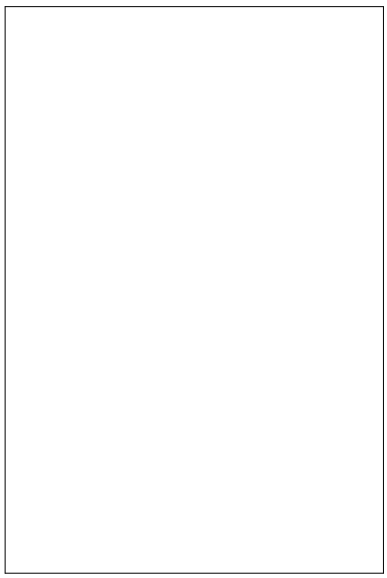

Fig. 2. Comparison between the transgenic (left) and the wild-type (right) tobacco plants. The transgenic plant (left) showed thickened stem and darker green color than the wild-type plant (right) did.

In general, the transgenic plants were very healthy and flowered normal. The transgenic tobacco plants showed an altered morphology in stem development by thickening about two-folds. This trait can be useful in a biotechnological aspect where the plant is exposed to high-wind environment and thereby is survived by the thick stem. Number of leaves was increased with reduced internode length and the leaves showed darker green (chlorotic) than the ones of wild-type plant did, suggesting that cytokinin overproduction in plant increases chloropyll content. Most of the transgenic plants that showed the altered phenotypes underwent severe wilting within hours after watering, indicating that the plants had high transpiration rate. This trait is also useful where water should be removed from the watery-soil. The results showed that high level of cytokinin influences the vegetative growth in the plants. The transgenic plant, however, had a poorly developed root system (data not shown). These results were consistent with the previous report (Li *et al.*, 1992) except for the stem thickness.

The observations suggest that the growth and developmental changes in the vegetative organs that occur upon overproduction of cytokinin in the transgenic plants result from critical concentration of cytokinins or possibly critical ratios of cytokinins to auxin at specific sites in the plants. The transgenic plants seems to contain high level of chlorophyll content in leaves as previously reported. It is also possible that the high level of cytokinin effects on the secondary metabolic pathway involved in other gene expressions such as defense related genes in the transgenic tobacco plants. It is necessary to pursue further physiological and molecular

analyses to understand metabolic changes in growth/development and to investigate inducible gene expressions by the cytokinin that involved in plant defense mechanism.

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