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Differential Expression of *Arabidopsis Cab* Promoters in Organs of Transformed Tobacco Plants

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형질전환된 담배식물체의 기관에 따른 *Arabidopsis Cab* Promoter의 차별적 발현

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ABSTRACT

Differential expression of the three chlorophyll a/b binding (cab) protein gene (*cab1*, *cab2*, and *cab3*) promoters of *Arabidopsis thaliana* was studied in tobacco plants transformed with *cab*-CAT (chloramphenicol acetyltransferase) translational fusions. CAT activity was measured to monitor the activities of the *cab* promoters. The activity of *cab1* promoter was higher than the other two in transformed tobacco leaves and also in calli and shoots derived from the leaves. Their activities were organ-specific and were the lowest in roots, medium in stems, and the highest in leaves. The relative activity of *cab1* promoter in stems comparing to it activity in leaves was, however, much higher than the values of *cab2* and *cab3*. When the *cab* promoter activity was expressed as CAT activity per unit chlorophyll instead of CAT activity per unit protein, the relative *cab1* promoter activity (stem/leaf) became almost unity. This result suggests that *cab2* and *cab3* show photosynthetic organ-specificity but *cab1* does not. Similar result was obtained in the differentiation process of stems and leaves from shoots derived from the transgenic tobacco leaves.

INTRODUCTION

Light-harvesting chlorophyll a/b binding (cab) proteins are the major components of the antenna complexes that capture and transfer light energy to the reaction centers of photosystem I and photosystem II (Gregory, 1989). *Cab* proteins are encoded by nuclear DNA and synthesized as larger precursors on cytoplasmic ribosomes which are imported into the chloroplasts, processed to their mature size and inserted into the thylakoid membranes (Cashmore, 1984; Jansson *et al.*, 1990; Viitanen, *et al.*, 1988).

The nuclear genes encoding the cab polypeptides have been cloned from a number of angiosperm species and are present in all plant species examined as a multigene family with 7-16 coding sequences for cab proteins. However *Arabidpsis thaliana* has only three (or four) *cab* genes, which are clustered within a 6.5 kb nuclear chromosomal region and code for the same mature protein (Leutwiler *et al.*, 1986). One of the three gene (*cab1*) shows less homology with the other two genes (*cab2* and *cab3*) in the 5′ untranslated regions of the gene (Ha and An, 1988; Mitra *et al.*, 1989).

In an effort to measure the independent expression

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of each *cab* promoter, An (1987) constructed *cab*-CAT translational fusions, but cab promoters were almost equally active in transformed calli or shoots derived from leaves. Leutwiler *et al.* (1986) reported that *cab1* was less active than the other *cab* genes in *A. thaliana*. Millar and Kay (1991) also reported that mRNA levels of the *cab2* and *cab3* genes showed a dramatic circadian cycling in both tobacco plants with *cab*-CAT translational fusions and *A. thaliana* shifted from light/dark cycles to constant darkness whereas the *cab1* mRNA level exhibited little or no cycling under the same conditions. However in cultured tobacco cells *cab3* activity was stronger than the other two (An, 1987).

These findings suggest that *cab* genes are differentially regulated in plants. An (1987) showed that *cab* promoter activities in green tissues were much stronger than in other tissues and Harkins *et al.* (1990) also reported that *cab* genes are expressed only in photosynthetic cells. In this study we examined further possible differential or photosynthetic organ-specific expressions of the three *cab* promoters and here we report that *cab2* and *cab3* show photosynthetic organ-specificity but *cab1* does not.

MATERIALS AND METHODS

Plant Materials. Tobacco plants (*Nicotiana tobaccum* cv. Xanti) were grown in a growth chamber at 25℃ under continuous light. T1 seeds pooled from transformed tobacco plants carrying the *cab*-CAT translational fusions were kindly donated from Dr. G. An (Washington State Univ., USA) and were grown in sterile culture on MS medium (4.3 g/*l* MS salts, 1 ml/*l* B_5 mixtures, 3% sucrose, 0.8% agar) (Murashige and Skoog, 1962) containing 200 mg/*l* kanamycin at 25℃ under continuous light for 2 weeks. For the induction of shoots, tobacco leaves were cut into about 0.1-0.5 cm^2 pieces. The plant sections were, then, wounded with forceps and were grown on a shoot induction MS agar medium containing 200 mg/*l* kanamycin, 1 mg/*l* 6-benzylaminopurine, 100 μg/*l* α-naphthalene acetic acid at 25℃ under dark condition. The etiolated shoots were transferred to a chamber under light condition for induction of stems and leaves.

Transformation of Plants. Tobacco plants were also transformed with *cab1* and *cab2* translational fusions according to An (1986) and the newly induced callus and shoots were used in this experiment to confirm the results obtained with the samples described above. *Cab*-CAT translational fusions (pGA568 and pGA570 with *cab1* and *cab2* promoters, respectively) were transferred into *E. coli* strain, MC1000 by the $CaCl_2$ method (Sambrook *et al.*, 1989). *Agrobacterium tumefacience* strain LBA4404 cells were transformed by the freeze/thaw method (An *et al.*, 1988) and were analyzed by a quick-screen method (Ebert *et al.*, 1987) based on the *E. coli* alkaline lysis procedure. Tobacco plants were transformed by leaf disc method (Horsch *et al.*, 1988).

Chloramphenicol acetyltransferase (CAT) assay. One gram of plant material was homogenized in 1 m*l* plant extraction buffer (0.5 M sucrose, 0.1 M Tris-HCl, 0.1% ascorbic acid, 0.1% cysteine-HCl). The lysate was centrifuged in an Eppendorf centrifuge for 5 min. An aliquot from each extract containing 20 μg of protein was used for CAT assay as described by An (1987). After finishing thin layer chromatography, separated spots of chloramphenicol and its acetylated forms were visualzed by autoradiography and quantified by liquid scintillation counting. The amount of acetylated chloramphenicol, in relation to the total amount of recovered ^{14}C is given as percent conversion rate. For the calculation of CAT activity per unit chlorophyll, total chlorophyll (chl) concentration was measured according to Arnon (1949) after extracting chl by dissolving an aliquot of the first supernatant in 80% acetone.

Chlorophyll fluorescence induction kinetics. Fluorescence induction kinetics were directly measured from leaves or shoots of tobacco plants using PAM chlorophyll fluorometer (Walz Co., Germany). Data were collected with a data acquisition board (DAS16G, Metrabyte Co., USA) installed in a IBM compatible personal computer and handled with computer programs written in Basic and Assembly languages.

RESULTS AND DISCUSSION

Selection and identification of transformed plants. Seeds from transgenic tobacco plants (T1 generation) with three different *cab*-CAT translational fusion genes were selected on MS agar medium containing kanamycin. About 75% of the seeds showed kanamycin resistency, which shows that the *cab*-CAT chimeric genes are segregated as a dominant Mendelian trait. Most of the selected plants (T2 generation) showed CAT activities and used for the following experiments.

Differential activation of *cab* promoters. Leaves of transformed tobacco plants with different *cab* promoters showed differences in their *cab* promoter activities (Fig. 1). The expression of *cab1* promoter was the highest and that of *cab3* promoter was the least.

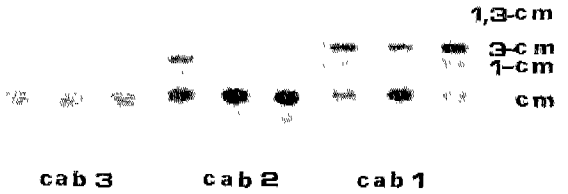

Fig. 1. Variation in *cab* promoter activities in leaves from independently transformed tobacco plants. cm, chloramphenicol; 1-cm, 1-acetyl chloramphenicol; 3-cm, 3-acetyl chloramphenicol; 1,3-cm, 1,3-acetyl chloramphenicol.

Variation in *cab* promoter activities. As shown in Fig. 1, variations in *cab* promoter activities were observed among T2 plants transformed with the same chimeric gene. Because of the random integration of the chimeric gene, this variation of the *cab* promoter activities may be due to the position effects by adjacent plant DNAs (An, 1986, 1987).

To study differential expression of the *cab* promoters less variant plants were required. Therefore upper stems with 3-4 leaves from each of the transgenic plants were cut and rooted in pots filled with vermiculites. Among clones asexually propagated in this way, the level of the variation in *cab1* promoter activities could be reduced (Table 1). Similar results were also obtained in the variations in *cab2* and *cab3* promoter activities (data not shown). *Cab1* promoter activity was about 2 times higher than that of *cab2* and *cab3* activity was lower than the value of *cab2* in most of the cases. The differences between the activities of *cab2* and *cab3* may be due to this position effect or it may be due to their intrinsic differences although *cab2* and *cab3* are more closely related compared to *cab*1 in their promoter sequences (Mitra *et al.*, 1989). An (1987) also reported that *cab3* promoter activity was the highest in calli derived from a suspension cultured tobacco line, whose ability to regenerate into whole plants has been lost.

Leaf-age dependent expression of *cab* promoters. The *cab* promoter activities in leaves of transformed plants showed age-dependency (Table 2). The activities from young plants were higher than those from aged plants and the activities in upper leaves were higher than those of lower leaves from the same plant. Therefore upper leaves from asexually propagated young plants with less than 10-12 leaves were used for later experiments.

Differential expression in shoots and calli. As shown above, the *cab1* promoter activity was much higher than the other two in transformed tobacco leaves. This difference is much more pronounced than that reported in An (1987). He observed similar results but with only minor differences in their expressions in calli or shoots derived from tobacco leaves. In our experiment, however, *cab1* promoter activities were much higher than the other two in both shoots and calli derived from transgenic tobacco leaves (Fig. 2). The differences in the activities of three *cab* promoters in calli and shoots are similar to those observed among the original leaves from which calli and shoots were generated.

Organ specificity of *cab* promoters activities. Activities of *cab* promoters showed organ-specificity similar to the report by An (1987) and Lamppa *et al.* (1985). Activities were much stronger in green leaves than stems (Fig. 3) and were negligible in roots (conversion rate=0.4-0.5 %). However the relative expression (stem/leaf) of *cab1* was 60-90%, which is much higher than the values of *cab2* and *cab3* (Table 3). Because the relative chlorophyll concentration to the protein concentration in stem tissues would be lower than the value in leaf tissues, we expressed the cab promoter activity as CAT activity per unit chlorophyll instead of those values per unit protein and the relative expression (stem/leaf) of *cab1* became nearly

Table 1. Variation of *cab1* promoter activities in transgenic tobacco plants

Plants from different seed	a	b	c			d		
Asexual clones			c1	c2	c3	d1	d2	d3
Conversion rate(%)	40.4	17.9	82.2	70.2	86.6	88.4	85.7	81.8
Mean ±1 Standard Deviation from Asexual clones			79.6± 8.5			85.3± 3.3		
Different seed[2]			55.8± 32.2					

[1]The cab promoter activity was expressed as units of percent acetylation of ^{14}C chloramphenicol.
[2]Mean values of the asexual clones were used for this calculations.

Table 2. Age dependent activities of three *cab* promoters in transgenic tobacco plants[1]

	Cab promoters		
	cab3	*cab2*	*cab1*
Young plant			
Upper leaf	40.4(100)[2]	40.8(100)	78.6(100)
Lower leaf	2.4(6)	37.8(92)	71.4(91)
Aged plant			
Upper leaf	11.3(100)	3.0(100)	48.0(100)
Lower leaf	1.6(14)	1.5(50)	5.8(15)

[1]The *cab* promoter activity was expressed as units of percent acetylation of ^{14}C chloramphenicol. [2]Values in parenthesis are relative activities.

unity (Table 3). This result suggests that *cab1* promoter does not show photosynthetic organ-specificity in stems and leaves of transgenic tobacco plants unlike to the other two promoters.

Differentiation of stems and leaves. After the onset of illumination (LD0: day 0 under light), the greening of dark-grown shoots and their differentiation into leaves and stems could be observed. First leaflets at the tip of the shoots became yellow green at LD1-LD3. Shapes of leaves could be distinguished and leaves became green gradually after LD3 and true leaves were observed after LD8 and evident at LD16. As shown in Fig. 4, cab activities in leaflets (or in leaves) increased as the time proceeded after the onset of the greening process but the increase in *cab2* and *cab3* activities in stem parts was much slower than that of *cab1*. Although stem parts of plantlets even at LD16 had less chlorophyll contents than leaf parts, there were no distinct differences in their appearance. Fluorescence rise patterns from the stem- and leaf-parts of the shoots after the onset of illumination suggest that the photosynthetic apparatus in stem-parts of the shoots are less developed compared with those of leaf-parts and no distinct differences in the photosynthetic machineries of shoots were observed among the transgenic plants with three different *cab* promoters (Fig. 5). These results suggest that *Arabidopsis cab* promoters are differentially regulated in the differentiation process of transgenic tobacco plants.

All the three *cab* promoters have TATA box and CCAAT box which are necessary for their light-dependent and tissue-specific promoter activities (Mitra *et al.*, 1989; Ha and An, 1988). In these papers they use the term "tissue-specificity" or "organ-specificity" when only photosynthetic tissues or organs show activities. The *cab1*

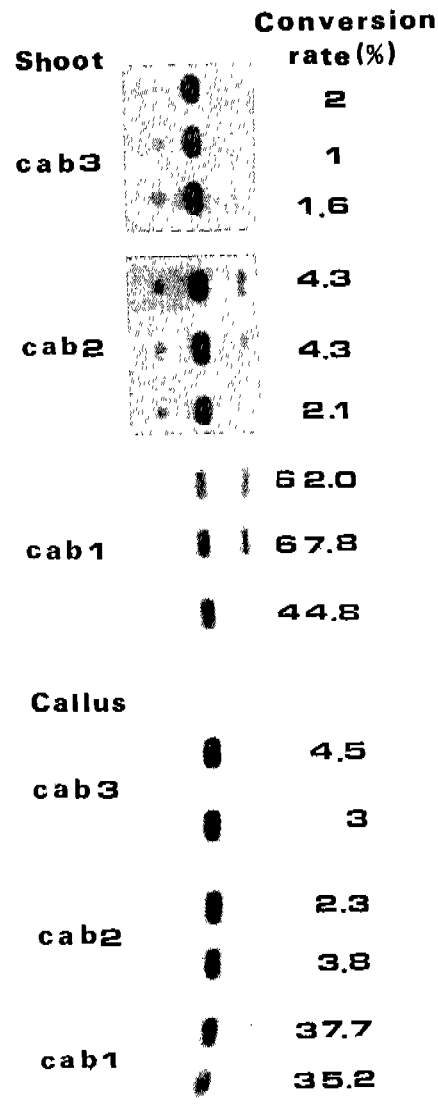

Fig. 2. *Cab* promoter activities in shoots and calli derived from transgenic tobacco leaves.

promoter has three G-box sequences whereas the other two have only one. The *cab1* promoter has an additional, potential Z-DNA forming sequences (An, 1987). Therefore our results can be explained as follows: the repression of *cab2* and *cab3* activities in stems is controlled by binding of a nuclear protein factor at the one G-box and one of the four controlling sites of the *cab1* promoter is responsible for the release of the repression. Millar and Kay (1991) reported that the *cab1*-CAT fusion did not show a strong circadian oscillation in the LD cycle but *cab2*-CAT fusion did in transformed tobacco plants.

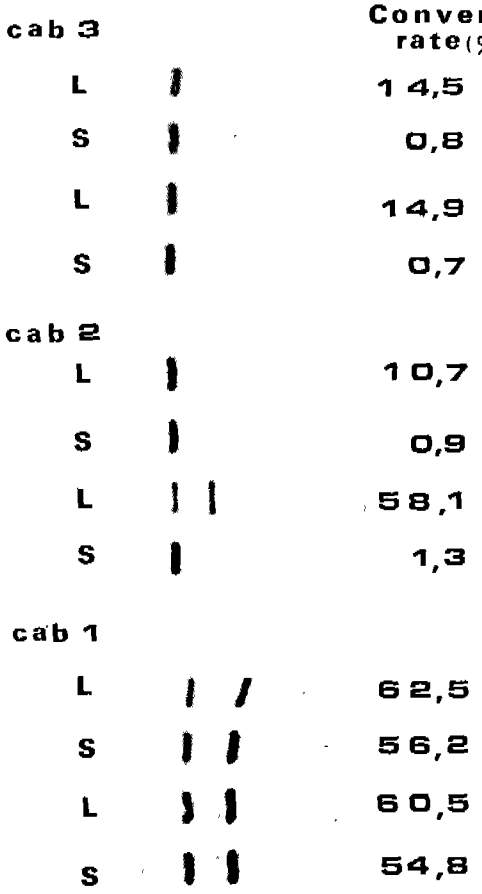

Fig. 3. Photosynthetic organ-specific expression of *cab* promoters in transgenic tobacco plants. L, leaf; S, stem.

The *cab3* promoter was also unable to activate a non-functional *nos* promoter whereas a similar *cab1-nos* promoter fusion was functional in the photosynthetic cells (Mitra *et al.*, 1989).

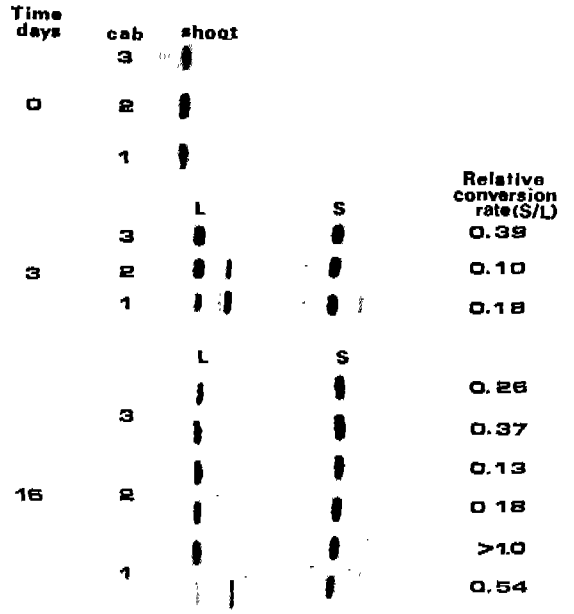

Fig. 4. Photosynthetic organ-specific expression of *cab* promoters during the differentiation of stems and leaves from shoots derived from trnasgenic tobacco plants. L, leaf; S, stem.

적 요

담배 식물체에서 *Arabidopsis thaliana*에 존재하는 3가지 다른 엽록소 a/b 결합 단백질 (*cab*) 유전자 promoter들의 발현을 조사하였다. 이들 promoter의 활성은 이들 promoter에 결합된 reporter gene의 산물인 CAT (chloramphenicol acetyltransfer- ase)의 활성으로 측정하였다. 담배엽에서 *cab* promoter의 활성은 *cab1, cab2, cab3*의 순이었고, 이들 잎에서 유도한 callus나 shoot에서도 동일한 양상을 보였다. 이들 3가지 promoter의 발현은 상부엽에서 하부엽보다 높은 활성을 보였으며 개체간의 높은 변이폭은 뿌

Table 3. *Cab* promoter activities in stems and leaves of transgenic tobacco plants expressed in two different ways

		Cab promoters					
		cab3		*cab2*		*cab1*	
CAT activity per unit protein (conversion rate)	Leaf	62.5	60.5	10.7	58.1	14.5	14.9
	Stem	56.2	54.8	0.9	1.3	0.8	0.7
	Ratio(%)	90	91	8	2	6	5
CAT activity per unit total chl (conversion rate)	Leaf	181	171	18	306	28	37
	Stem	264	202	4	20	5	4
	Ratio(%)	>100	>100	19	7	18	12

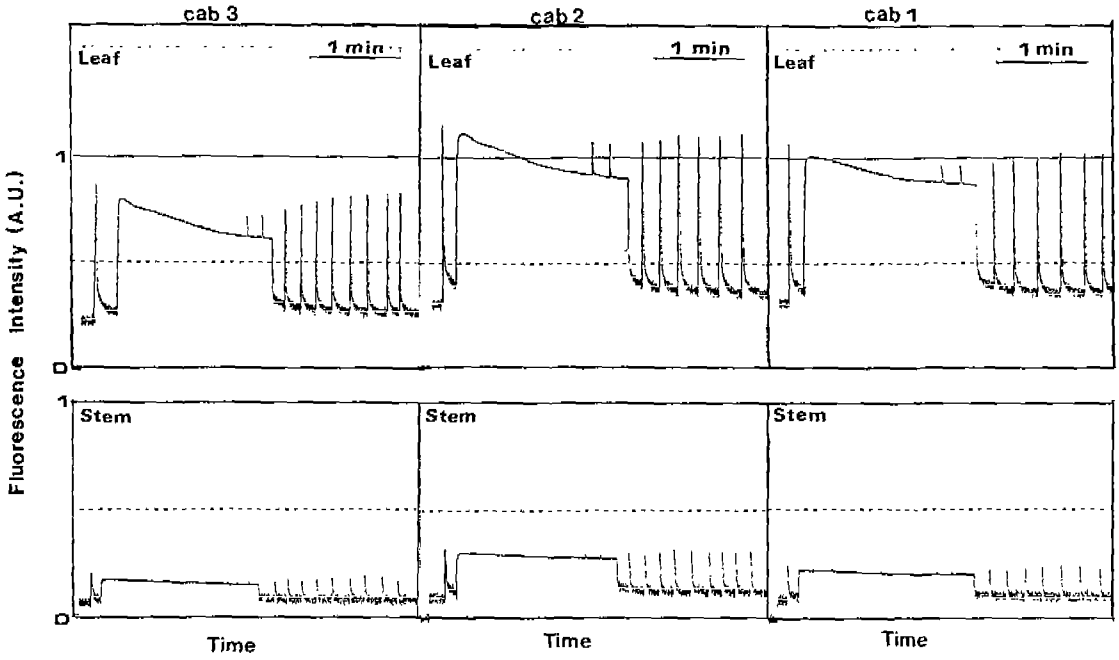

Fig. 5. Fluorescence induction kinetics of stem- and leaf-parts of the dark grown shoots of transgenic tobacco plants at the day 16 after the onset of illumination.

리내리기를 통하여 무성증식된 식물체를 사용하여 줄일 수 있었다. 이들은 또한 기관 특이성을 보여 줄기 보다 잎에서의 활성이 높았고 뿌리에서는 거의 발현 되지 않았다. 그러나 *cab1* promoter의 경우, 다른 두 *cab* promoter들과는 다르게, 잎에 대한 줄기에서의 상대적인 활성이 높았으며, CAT 활성을 단위 단백질당을 표시하는 대신 단위 엽록소당 활성으로 나타내었을 때 줄기와 잎에서 활성의 차이가 관찰되지 않았다. 즉 *cab2*와 *cab3*는 광합성 기관 특이성을 보이는 반면 *cab1*의 경우는 이러한 특이성을 나타내지 않았다. 이러한 현상은 잎에서 유도한 shoot가 줄기와 잎으로 분화되는 과정에서도 관찰되었다.

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