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Differential Activation of *Arabidopsis Cab* Promoters during Greening of Transgenic Tobacco Shoots

Lee, Choon-Hwan, Yejeong Ko, Hong-Keun Choi*, Tae Hyong Rhew and Chin Bum Lee*****

*(Department of Molecular Biology, Pusan National University, Pusan, *Department of Biological Science, Ajou University, Suwon, **Department of Biology, Pusan National University, Pusan, and ***Department of Biology, Dongeui University, Pusan)*

형질전환된 담배 어린 싹의 녹화과정 중 *Arabidopsis Cab* 프로모터의 활성 차이

李 春 煥·高 禮 貞·崔 鴻 根*·柳 兌 馨**·李 鎭 範***

(부산대학교 분자생물학과, *아주대학교 생명과학과, **부산대학교 생물학과, ***동의대학교 생물학과)

ABSTRACT

Promoters of the chlorophyll a/b binding protein genes, *cab1,* and *cab2,* of *Arabidopsis thaliana* were studied for their functions in differential expression during greening of etiolated shoots. The etiolated shoots were derived from leaves of transgenic tobacco plants with the *cab*-CAT (chloramphenicol acetyltransferase) translational fusions, and CAT activity was measured to monitor the activities of the *cab* promoters. *Cab1* promoter activity increased rapidly and showed saturation after about 24 hours of greening, but that of *cab2* increased with about 2 day-lag period and showed saturation after 6 days. *Cab1* promoter activity was more sensitive to levulinic acid (LA) compared with *cab2* activity. *Cab2* promoter activity was inhibited more sensitively by chloramphenicol (CAP) than by inhibitors of Chl formation. *Cab1* promoter activity was, however, inhibited less sensitively by CAP than by LA. The treatment of abscisic acid (ABA) did not block Chl synthesis so significantly as LA treatment did, and *cab2* promoter activity was much less sensitive to ABA compared with that of *cab1.* These results suggest that *cab1* expression is strongly related with Chl formation, possibly with δ-aminolevulinic acid accumulation, and *cab2* expression is suppressed more by the blockage of translation of Chl a-apoproteins than by the bloackage of Chl a accumulation.

INTRODUCTION

Chlorophyll a/b binding (*cab*) proteins are encoded by a family of nuclear genes, synthesized as larger precursors on cytoplasmic ribosomes which are imported into the chloroplasts, processed to their mature size, and inserted into the thylakoid membranes together with chlorophyll and some proteins encoded by chloroplast genes (Chitnis and Thornber, 1988). *Arabidpsis thaliana* has only three (or possibly four) *cab* genes, which are clustered within a 6.5 kb nuclear chromosomal region and code

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for the same mature protein (Leutwiler *et al.*, 1986). One of the three genes (*cab1*) shows, however, 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).

The expression of these genes is light-regulated and organ-specific (An, 1987; Ha and An, 1988; Mitra *et al.*, 1989). Harkins *et al.* (1990) also reported that *cab* genes are expressed only in photosynthetic cells. Differential expression of these two groups of *cab* genes due to their differences in 5' untranslated regions has been reported. In our previous paper (Hong *et al.*, 1992), *cab2* and *cab3* showed photosynthetic organ-specificity, but *cab1* did not both in tobacco plants and in developing shoots derived from their leaves. Millar and Kay (1991) also reported that mRNA levels of *cab2* and *cab3* genes showed a dramatic circadian cycling after shifted from light/dark cycles to constant darkness both in *A. thaliana* and in tobacco plants with *cab*-CAT translational fusions, whereas the *cab1* mRNA level exhibited little or no cycling under the same conditions. 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).

Photosystem II is composed of *cab* proteins, Chl, and some chloroplast-encoded proteins. It is quite natural to assume that *cab* gene expression is regulated by Chl synthesis and/or by the synthesis of chloroplast-encoded proteins. Sun and Tobin (1990) reported that mRNA levels of the *cab1* gene was 6 times higher than those of the other two genes during greening of etiolated *A. thaliana* (strain Columbia) seedlings.

In this study, possible differential expressions of the two groups of *cab* promoters during greening were examined and the results showed that expression patterns of *cab1* and *cab2* promoters were different and they were differently inhibited by inhibitors for Chl synthesis and plastid-coded protein synthesis during greening of etiolated shoots derived from transgenic tobacco leaves with the *cab*-CAT translational fusions.

MATERIALS AND METHODS

Plant materials. Tobacco plants (*Nicotiana tobaccum* cv. Xanti) were grown in a growth chamber at 25℃ under continuous light. *Agrobacterium*-mediated transformaton of plants with *cab*-CAT translational fusions (pGA568 and pGA570 with *cab1* and *cab2* promoters, respectively) and their asexual propagation were performed as described previously (Hong *et al.*, 1992). Seeds of transformed tobacco plants (T1 generation) carrying *cab*-CAT translational fusions were grown sterile on MS agar medium (Murashige and Skoog, 1962) containing 500 mg/L kanamycin at 25℃ under continuous light for 6 weeks.

Induction of etiolated shoots. For the induction of etiolated 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 MS agar medium for shoot induction containing 500 mg/L kanamycin, 1 mg/L 6-benzylaminopurine, and 100 μg/L α-naphthalene acetic acid at 25℃ under dark condition for about three weeks.

Treatment of chemicals. Dark-grown etiolated shoots were transferred to a medium containing 40 mM levulinic acid (LA), 100 μM ABA, or 1.24 mM chloramphenicol (CAP) 18 hours before the onset of illumination. If there is no special mention, all chemicals were purchased from Sigma Chemical Co.

Chloramphenicol acetyl transferase (CAT) assay. The CAT activity was determined as described by Pröls *et al.* (1988). Plant materials were homogenized in 1 mL/g of 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 was incubated with reaction buffer (0.25 M Tris-HCl (pH 7.8), 10 μL of 4 mM acetyl-CoA, 0.5 μL of ^{14}C-chloramphenicol (Dupon Co.)) at 37℃ for 45 min. The reaction was terminated by addition of 1 mL ethylacetate. After centrifugation, the supernatants were evaporated at 96℃. Dry pellets were dissolved in 20 μL of ethylacetate and submitted to ascending chromatography in chloroform/methanol (95 : 5, v/v) on silica gel plates (Eastman Kodak Co.). Separated spots of chloramphenicol and its acetylated forms were visualized by autoradiography and quantitated by liquid scintillation counting. The amount of acetylated chloramphenicol, in relation to the total amount of recovered ^{14}C was given as percent conversion rate.

Chl and protein analyses. The first precipitates described above were resuspended in 80% (v/v) aqueous acetone, and total Chl contents per gram fresh weight were determined according to Arnon (1949). Soluble protein contents were determined as described by Lowry *et al.* (1951).

Chl fluorescence induction kinetics. Fluorescence induction kinetics were measured directly from the leaf parts detached from tobacco shoots using PAM chlorophyll fluorometer (Walz Co., Germany). Data were collected with a data acquisition board (DAS16G, Metrabyte Co.,

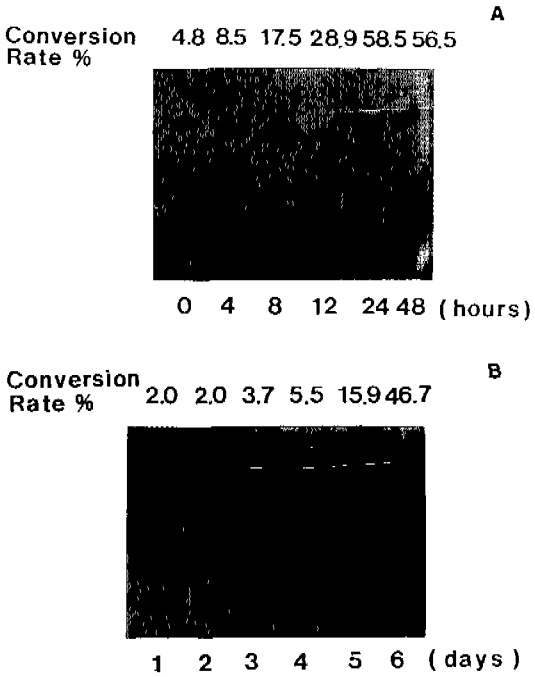

Fig. 1. Changes in *Arabidopsis cab* promoter activities during greening of etiolated tobacco shoots. Tobacco shoots carry (a) *cab1*-CAT or (b) *cab2*-CAT translational fusions.

USA) installed in a IBM compatible personal computer and handled with computer programs written in Basic and Assembly languages.

RESULTS

Activities of *cab* promoters during greening. Although transgenic tobacco plants with *cab1*-CAT or *cab2*-CAT translational fusion genes and the shoots generated from their leaves showed kanamycin resistance, it was not easy to test whether a specimen carries *cab1* promoter or *cab2* promoter. In our previous report (Hong *et al.*, 1992), *cab2* promoter activities in stems were much lower than those in leaves, but *cab1* promoter activities were similar both in leaves and in stems. Therefore, leaves and stems from plants or light-grown shoots were tested for their relative (stem/leaf) CAT activities to ensure their identities (data not shown).

Changes in *cab1* and *cab2* promoter activities of etiolated tobacco shoots after they were exposed to continuous light are shown in Fig. 1(a) and (b), respectively. The conversion rates listed in these figures are the means

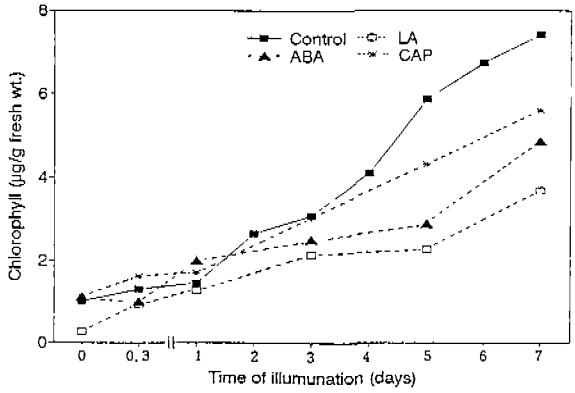

Fig. 2. Changes in levels of chlorophyll during greening of etiolated tobacco shoots treated with LA, ABA or CAP.

of three independent measurements. *Cab1* promoter activity was very low at the onset of illumination, increased rapidly afterwards, and showed saturation after 20~24 hours. However, the activity of *cab2* promoter did not increased promptly after illumination but seemed to require 1 or 2 days of lag-period, because *cab2* activity was detectable after 2~3 days of illumination. *Cab2* promoter activity showed saturation after 6 days of illumination.

Effects of LA and ABA on activities of *cab* promoters. To test the relationship between *cab* gene expression and chlorophyll synthesis, an inhibitor of Chl synthesis, LA, was treated on the growth medium of shoots 18 hours before the start of greening. When dark-grown shoots were pretreated with LA, Chl synthesis was inhibited significantly as shown in Fig. 2.

By the treatment of LA, *cab1* promoter activity was decreased almost completely (Fig. 3(a)), but *cab2* promoter activity was reduced by about 50% at 7D (Fig. 3(b)). ABA, a regulator for plant growth and development, is known to affect Chl formation ability (Sundqvist, 1978). By the treatment of ABA, Chl contents were reduced significantly (Fig. 2). *Cab1* promoter activity was also decreased significantly (Fig. 4(a)) but not completely as shown in LA-treated samples (Fig. 3(a)). *Cab2* promoter activity was reduced by about 25% at 7D (Fig. 4(b)).

Effects of CAP on activities of *cab* promoters. When the formation of plastid-encoded thylakoid membrane proteins was inhibited by the treatment of CAP on the growth medium of shoots 18 hours before the start of greening, *cab* promoter activities were decreased much more significantly (Fig. 5) than the accumulation of chlorophyll in the shoots did (Fig. 2). It was notable that

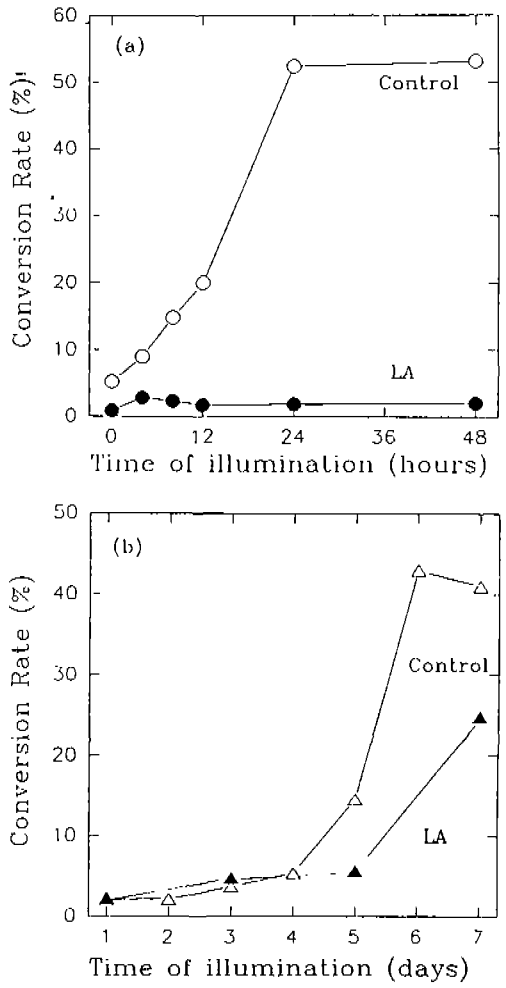

Fig. 3. Effect of LA on *Arabidopsis cab* promoter activities during greening of etiolated tobacco shoots. Tobacco shoots carry (a) *cab1*-CAT or (b) *cab2*-CAT translational fusions.

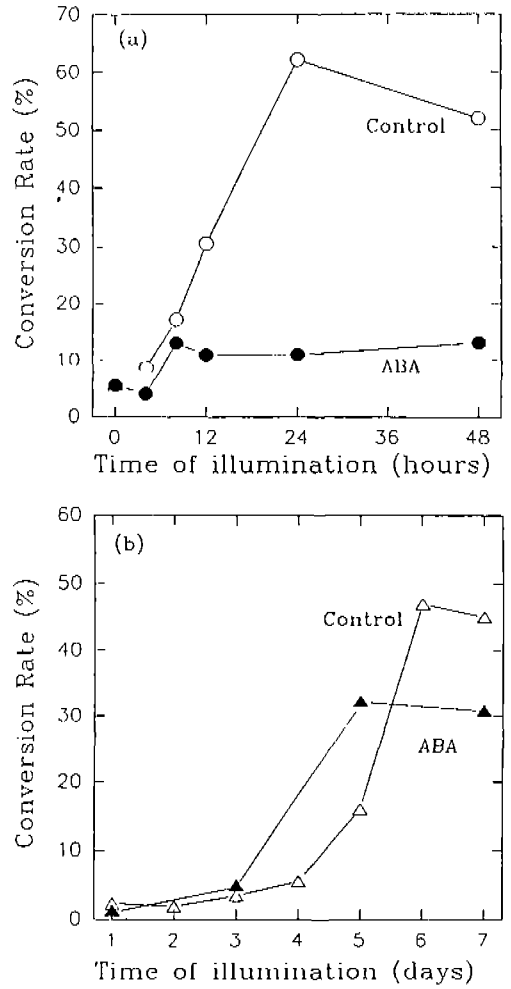

Fig. 4. Effect of ABA on *Arabidopsis cab* promoter activities during greening of etiolated tobacco shoots. Tobacco shoots carry (a) *cab1*-CAT or (b) *cab2*-CAT translational fusions.

cab2 promoter activity was inhibited more by CAP than by the treatment of LA or ABA, and *cab1* promoter activity was inhibited less by CAP than by the treatment of LA (and ABA).

Effect of chemicals on photosynthetic apparatus. Tobacco shoots carrying two different *cab* promoters could not be distinguished by their appearances. In Fig. 6, the florescence induction profiles of control and chemical-treated shoots carrying *cab1* promoter after 1 day of illumination are shown. The profiles from shoots carrying *cab2* promoter was almost identical (plots not shown). The results confirm that there were no significant differences in the photosynthetic machineries between the tobacco shoots transformed with *cab1*-CAT fusion and those transformed with *cab2*-CAT. The same figure shows that the development of photsynthetic machinery in tobacco shoots was hindered significantly by the treatment of LA and ABA but not so significantly by the treatment of CAP.

DISCUSSION

The expression of *cab1* was very sentive to light. As shown in Fig. 1a, *cab1* promoter activity was very low at zero time, increased rapidly, and showed saturation after 20~24 hours of illumination during greening of

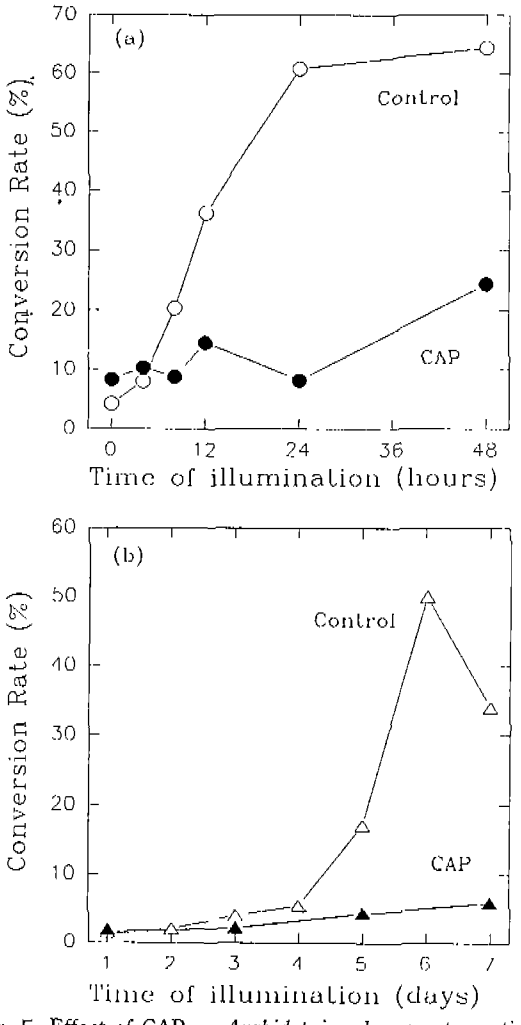

Fig. 5. Effect of CAP on *Arabidopsis cab* promoter activities during greening of etiolated tobacco shoots. Tobacco shoots carry (a) *cab1*-CAT or (b) *cab2*-CAT translational fusions.

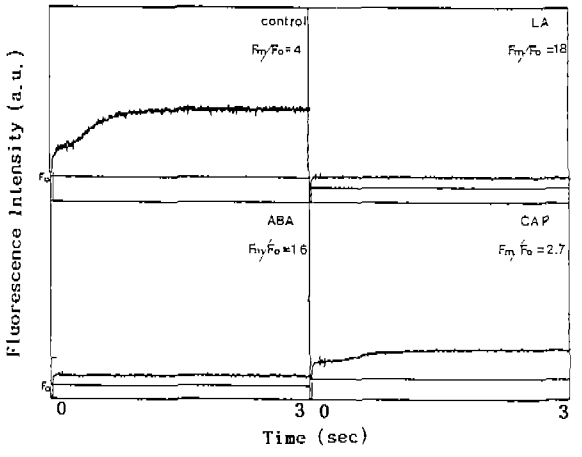

Fig. 6. Fluorescence induction kinetics from etiolated tobacco shoots, pretreated with LA, ABA or CAP, 1-day after the onset of illumination.

etiolated transgenic tobacco shoots. Sun and Tobin (1990) also reported that *cab1* mRNA level became six times higher than that of *cab2* plus *cab3* from etiolated Arabidopsis (strain Columbia) seedling after a brief red light treatment. *Cab1* promoter activity was much sensitive to LA compared with that of *cab2* (Fig. 3). LA is known to block chlorophyll synthesis as an inhibitor of ALA-dehydratase by competing with ALA in many photosynthetic organisms (Beale and Castelfranco, 1974; Johanningmeier and Howell, 1984). Chl synthesis was increased gradually for 7 days of greening period and inhibited significantly by the treatment of LA (Fig. 2).

Cab1 promoter activity might to be inhibited by the accumulation of ALA or activated by the synthesis of Chl. At the onset of illumination, *cab1* activity was less in the LA pretreated samples than in control (Fig. 3(a)). Possible explanation might lie on that the etiolated shoots were transferred to LA-containing media 18 hours before the onset of illumination, and they were briefly exposed to weak light from an alcohol lamp. During dark period, protochlorophyllide accumulates and requires light to form Chl, which can be converted back to protochlorophyllide under dark (Castelfranco and Beale, 1981). Therefore, the LA treated sample may contain less protochlorophyllide and more ALA compared with those of control. The possible inhibition of *cab1* transcription by accumulation of ALA is remained for further study.

By the treatment of ABA, Chl synthesis was also blocked but less significantly (Fig. 2). *Cab1* promoter activity was much more sensitive to ABA compared with that of *cab2* (Fig. 4). Although ABA is known to inhibit Chl formation, its precise role has not been well defined yet. However, Chang and Walling (1991) reported a reciprocal relationship between ABA and *cab* mRNA during soybean embryogeny. ABA negatively regulates *cab* gene expression, but its detailed mechanism is still uncertain.

The activity of *cab2* promoter increased with about 2 day lag-period and showed saturation after 6 days, which was very much different from the induction profile of *cab1* profile (Fig. 1). In addition, *cab2* promoter activity was inhibited more sensitively by CAP than by inhibitors of Chl formation, and *cab1* promoter activity was less

sensitive to the treatment of CAP than to the treatment of LA (Figs. 3~5). Klein *et al.* (1988) suggested that light-induced conversion of protochlorophyllide to Chl a is necessary for accumulation of the plastid-coded Chl a-apoprotein, which is controlled at a posttranscriptional level. The current results suggest that the expression of *cab2* gene is suppressed more by the blockage of translation of Chl a-apoproteins than by the blockage of Chl a accumulation.

적 요

담배잎에서 유도하여 암소에서 키운 어린 싹의 녹화과정 중, *Arabidopsis thaliana*에 존재하는 2가지 다른 엽록소 a/b 결합 단백질 유전자(*cab1*, *cab2*) 프로모터의 발현을 조사하였다. 이들 프로모터들의 활성은 프로모터에 결합된 CAT(chloramphenicol acetyltransferase) 유전자 산물의 활성으로 측정하였다. *Cab1* 프로모터의 활성은 광합하에서 급속히 증가하여 1일 정도 경과 후 포화치를 보였으나, *cab2* 프로모터의 활성은 약 2일 정도의 지연기를 가진 후 약 6일 후에 포화치에 도달하였다. Levulinic acid(LA) 처리에 의하여 *cab2*보다 *cab1* 프로모터의 활성이 크게 억제되었고, *cab2* 프로모터의 활성은 엽록소 합성억제제 보다 chloramphenicol(CAP)에 의하여 크게 억제되었으며, *cab1*은 그 반대로 나타났다. ABA 처리로, 엽록소 합성은 LA 처리 경우보다 적게 억제되었으며, *cab2* 프로모터의 활성의 억제 정도는 *cab1*에 비하여 훨씬 적었다. 이러한 *cab* 프로모터 간의 활성의 차이는 각 프로모터를 가지는 시료의 차이에 의한 것은 아니며, 광합성기구의 발달이 CAP의 처리 경우에 비하여 LA와 ABA의 처리에 의하여 크게 저해됨을 형광유도과정의 분석을 통하여 확인하였다.

이상의 결과로 보아 *cab1* 프로모터의 발현은 엽록소 합성과 크게 관련되어, 아마 δ-aminolevulinic acid의 축적에 의하여 조절되며, *cab2* 프로모터의 활성은 엽록소 a의 축적보다는 엽록소내의 엽록소 a-apoprotein의 합성과 관련되어 조절되는 것으로 사료된다.

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