*Plant Biotechnology* 25, 573–577 (2008)

**Short Communication**

# Suppression of carotenoid synthesis in transgenic *Arabidopsis* cultured cells over-expressing the *AHL29/SOB3* gene

Ryosuke Sano[1], Hideyuki Suzuki[1], Yoichi Ogawa[1], Tomoko Dansako[1,a], Nozomu Sakurai[1], Koei Okazaki[1], Koh Aoki[1], Kazuki Saito[2], Daisuke Shibata[1,*]

[1] Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan; [2] Graduate School of Pharmaceutical Sciences, Chiba University, Inage, Chiba 263-8522, Japan
* E-mail: shibata@kazusa.or.jp Tel: +81-438-52-3947 Fax: +81-438-52-3948

Received September 19, 2008; accepted October 21, 2008 (Edited by M. Umeda)

**Abstract** Up-regulation of the *Arabidopsis* gene *AHL29/SOB3* (*At1g76500*), encoding a protein with an AT-hook DNA-binding protein motif, is known to function as a suppressor of the *phyB* phenotype and to cause delayed senescence. We over-expressed the full-length cDNA of *AHL29/SOB3* under the cauliflower mosaic virus 35S promoter in *Arabidopsis* suspension-cultured T87 cells. Preliminary DNA array experiments suggested down-regulation of many genes of the carotenoid synthesis pathway in the transgenic cells. Metabolite analysis with a liquid chromatography-coupled mass spectrometer demonstrated that the accumulation of the carotenoids lycopene, $\alpha$-carotene, $\beta$-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin was suppressed in the transgenic cells. These results suggest that up-regulation of *AHL29/SOB3* down-regulates the mechanism of carotenoid biosynthesis at the transcriptional level.

**Key words:** *Arabidopsis* T87 cell line, AT-hook motif protein, carotenoid synthesis, *phyB*, senescence.

Up-regulation of transcription factor genes in plants is a promising way to control metabolism or useful morphological traits, such as organ size, in plant biotechnology. Activation tagging is a gain-of-function mutagenesis method in which a strong enhancer element is introduced into chromosomes and should result in the activation of an adjacent gene. It has been used to isolate novel corresponding genes through screens for dominant traits. A large collection of activation tagging lines have been created using *Arabidopsis thaliana* as a host plant, and several genes have been isolated (Weigel et al. 2000). A Myb transcription factor gene, *production of anthocyanin pigment 1-Dominant* (*pap1-D*), was isolated from a bright-purple mutant found in a pool of ~5000 *Arabidopsis* activation tagged lines (Borevitz et al. 2000). The function of *pap1-D* was further characterized by integrated analysis of the metabolome and transcriptome (Tohge et al. 2005). Another useful gain-of-function mutagenesis method in plants is to up-regulate a gene by driving its full-length cDNA with a strong enhancer. As a large number of full-length cDNA clones of various plants including *Arabidopsis* (Seki et al. 2002), rice (Kikuchi et al. 2003), and tomato (Yano et al. 2006) are now available, the method is applicable to a great variety of genes, including transcription factor genes. Up-regulation of the transcription factor gene *Myb28* results in the production of large amounts of glucosinolates, which are known to provide anticarcinogenic, antioxidative and antimicrobial activities in *Arabidopsis*-cultured suspension cells which do not normally accumulate those metabolites (Hirai et al. 2007). In that study, the full-length cDNA of *Myb28* controlled under the cauliflower mosaic virus 35S promoter was introduced in the *Arabidopsis* cell line T87. Recently, co-expression network analysis has become prevalent in post-genomic research to relate transcription factor genes with a set of genes of interest, aiding the study of transcriptional regulatory mechanisms of these genes (for a review, see Aoki et al. 2007). Ogawa et al. (2008) developed a protocol for efficient and high-throughput vector construction using full-length cDNA clones and *Agrobacterium*-mediated transformation of *Arabidopsis*, which will facilitate functional genomics with gain-of-function technology.

DNA binding proteins with AT-hook motifs are thought to play a role in the regulation of transcription by affecting the architecture of chromatin. The motif interacts with the narrow minor groove of AT-rich DNA sequences (Aravind and Landsman 1998). These finding are mainly based on studies in animals and yeast, but

[a] Present address: Core Laboratory, Nara Prefectural small and medium-sized enterprises Support Corporation, 88 Shijo, Kashihara, Nara 634-0813, Japan

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knowledge of this protein in plants has accumulated recently. The *Arabidopsis* AT-hook motif proteins consist of approximately 30 members and can be largely classified into two phylogenetic groups (Fujimoto et al., 2004). Among them, the protein encoded by *At4g12080* has been characterized as a nucleoplasm localized protein and called AHL1 (AT-hook motif nuclear localized protein 1) (Fujimoto et al. 2004). From attempts to screen activation tagged lines for highly significant delays of leaf senescence and for suppressors of the long-hypocotyl phenotype of a weak *phyB* allele, *ORE7* (*At1g20900*) (Lim et al. 2007), and *ESC* and *SOB3* (*At1g76500*) were identified (Street et al. 2008), respectively, all of which are members of the AT-hook motif proteins with very similar amino acid sequences; *ESC* is identical to *ORE7* (hereafter *AHL27/ORE7/ESC* and *AHL29/SOB3*, respectively). *AHL27/ORE7/ESC* (Lim et al. 2007; Street et al. 2008) and *AHL29/SOB3* (Street et al. 2008) are localized in the nucleus. Previous studies of these genes show that they function redundantly as negative regulators of leaf senescence (Lim et al. 2007) and as negative modulators of hypocotyl elongation (Street et al. 2008) when up-regulated. Another close relative of *AHL27/ORE7/ESC* and *AHL29/SOB3*, AHL25 (At4g35390), named AGF1, was identified as a DNA-binding protein for the cis-acting sequence of gibberellin-negative feedback (Matsushita et al. 2007). In *Catharanthus roseus*, the *APETALA2*-domain transcription factor *ORCA3* is involved in the jasmonate-responsive activation of terpenoid indole alkaloid biosynthetic genes, and it has been suggested that an autonomous jasmonate-responsive element (JRE) within the *ORCA3* promoter is bound by AT-hook motif proteins (Vom Endt et al. 2007). As gibberellins and jasmonates are known to modulate metabolism, it is possible that up-regulation of genes encoding AT-hook motif proteins causes changes in metabolism. However, this has not been studied.

In our large-scale production of gain-of-function mutants with *Arabidopsis* full-length cDNA fragments under control of the 35S promoter, we up-regulated the *AHL29/SOB3* gene in suspension-cultured cells of the *Arabidopsis* line T87. In this study, we characterized the transgenic cell lines for metabolic changes.

As the original RAFL cDNA clone for *At1g76500* (RAFL15-12-P18) obtained from RIKEN BioResource Center (Tsukuba, Japan) was a chimeric clone at the 5′ UTR region of the target gene (the coding region of a certain gene on chromosome 5 was fused in head-to-head direction), we modified the RAFL clone by replacing the original erroneous 5′ region with the correct fragment amplified by RT-PCR. To build the corrected RAFL clone, the *Mun*I-*Bgl*II digested fragment amplified with a pair of PCR primers, AThk1G7-0-c5 (5′-TATACAATTGCTCTGTTTTTGTC-GGCTTGT-3′) and AThk1G7-2-c3 (5′-CTCCAGAT-GAGACTTCAAGAACAT-3′), by KOD -Plus- Ver.2 (TOYOBO, Osaka, Japan) was ligated into the original RAFL clone digested with *Eco*RI-*Bgl*II. The sequence of entire cDNA region was verified. The corrected RAFL clone was consequently cloned into the Gateway-based binary vector pGWB2 (having the CaMV 35S promoter to drive the target) and *Agrobacterium*-mediated transformation of *Arabidopsis* T87 cells was performed, as described previously (Ogawa et al. 2008). Twenty independent transgenic calli were isolated and subjected to northern analysis to confirm the up-regulation of the introduced gene. Preparation of T87 suspension cells, extraction of total RNA, and northern blot analysis were performed essentially as reported in Ogawa et al. (2008). Ten-day-old cell cultures following two weeks of preculture (in 100-ml flasks containing 30 ml of liquid medium with hygromycin and meropenem) were used as samples for RNA extraction. RNA samples of transgenic and wild-type T87 calli were extracted using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Probe DNA fragment was amplified with the full-length cDNA as a template, using a pair of oligonucleotides, 5′-TCCCTACTCTCGCGTTAACGCTAGC-3′ and 5′-GTAACATCAGAGATTTTGAGACAC-3′. Labeling of the probes and hybridization were performed using AlkPhos Direct Labeling and Detection System with CDP-Star according to the instruction manual (GE Healthcare, Buckinghamshire, UK). Five transgenic cell lines expressing the gene highly (Figure 1B) were used for further analysis. Our preliminary DNA array analysis (using Agilent Arabidopsis2 Oligo DNA Microarray; two-color procedure) utilizing KaPPA-View (Tokimatsu et al. 2005) suggested that many carotenoid biosynthetic genes were down-regulated in the transgenic lines. Thus, we subjected the cell lines to further analysis with metabolite changes.

We analyzed carotenoid contents of the transgenic cell lines using liquid chromatography coupled mass spectrometry (Figure 2, Table 1). Metabolites were extracted from 100 mg of frozen-stored cell samples with 5 volumes of a chloroform:methanol (3 : 1 [v/v]) solution by homogenization using a mixer mill (TissueLyser, Qiagen) at 25 Hz for 2 min twice. After centrifugation (~18000×*g*, 15 min at 4°C), supernatant was recovered and stored following filtration with a 0.2-$\mu$m PTFE filter (Millipore, Billerica, USA). Residual debris was saponified with 300 $\mu$l of 1 M methanolic KOH at 60°C for 30 min in darkness, following vigorous agitation. After mixing the resultant solution with 500 $\mu$l of $H_2O$, metabolites were extracted twice with 10 volumes of chloroform by vigorous agitation, followed by recovery and filtration as above. All of the filtrate was merged, evaporated to dryness, and the residue was re-dissolved in 150 $\mu$l of acetone and analyzed with ultraperformance

liquid chromatography coupled with a quadrupole Time-of-Flight mass spectrometer (UPLC-Q-TOF/MS). Chromatography was carried out on an Acquity UPLC system (Waters, Milford, USA) with a diode array detector. Throughout chromatography, the eluate was monitored continuously from 210 to 500 nm (Resolution: 1.2 nm; Sampling Rate: 20 points $s^{-1}$). An acetone extract (Injection volume: 5 $\mu$l) was applied to a Acquity UPLC BEH Shield RP18 column (2.1×150 mm, 1.7 $\mu$m; Waters) at a flow rate of 0.3 ml $min^{-1}$, and the column oven temperature was set at 40°C. The sample was analyzed by a gradient elution schedule based on Iijima et al. (2008) with some modification as follows: 3% acetonitrile (solvent A) and acetonitrile (solvent B) used as the mobile phase, 80% B to 100% B (15 min), 100% B (15 min), 100% B to 80% B (1.5 min), and 80% B (2.5 min). The APCI setting was as follows: Corona current was 3 $\mu$A, source and desolvation temperatures for positive-ionization mode were 90 and 350°C, respectively, and nitrogen sheath gas flow rate was set at 600 liters $h^{-1}$. The peaks were identified by comparing their specific retention times and absorption spectra with authentic standards. Each carotenoid was quantified by measuring the peak area value at 450 nm using a standard curve of the authentic compound. Among the

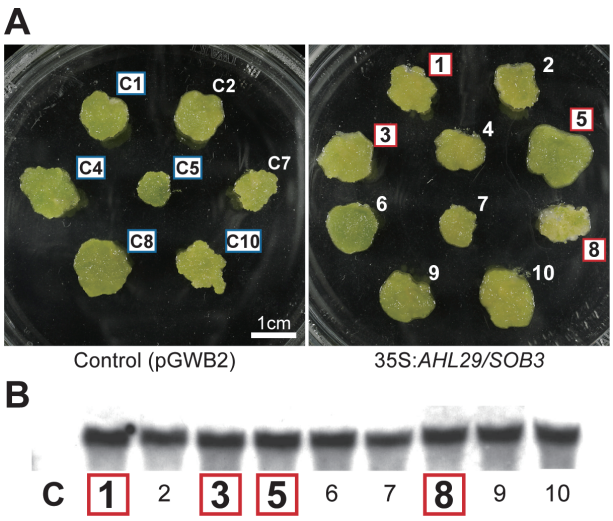


Figure 1. Photographs of calli used in this study and expression level of *AHL29/SOB3* in transgenic T87 cell lines. (A) Each individual callus of 35S: *AHL29/SOB3* (right) and control empty vector (left) transgenics with line number. Number with color frame denotes individual number of lines used for carotenoid analysis (5 lines each; one of the 35S: *AHL29/SOB3* lines is not shown). Note that all lines except #8 of 35S: *AHL29/SOB3* have green color comparable to control lines. (B) Northern blot analysis of individual callus lines shown in (A) (line #4 has no data). Lane 'C' denotes the mixture of total RNAs for all control empty vector lines shown in (A), and lane number with red frame indicates lines used for subsequent analysis. Note that control lines have no detectable signal and all 35S: *AHL29/SOB3* lines have comparable abundant expression.

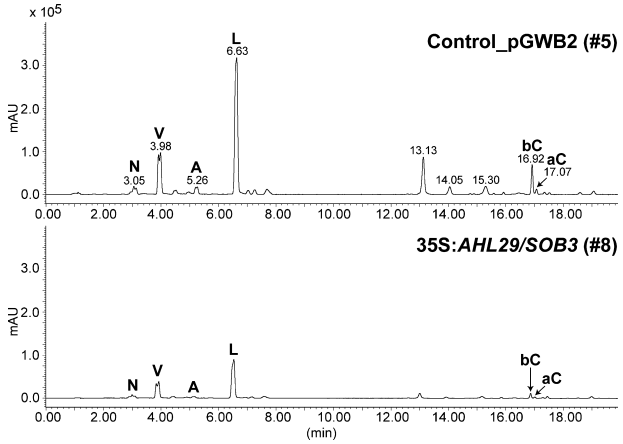


Figure 2. UPLC/PDA chromatograms of chloroform–methanol extracts of T87 suspension cells. Representative data of control vector (top) and 35S: *AHL29/SOB3* (bottom) transgenics are shown. Numbers and letters on peaks indicate Rt and abbreviation for identified carotenoids with authentic standards, respectively: N, Neoxanthin; V, Violaxanthin; A, Antheraxanthin; L, Lutein; bC, $\beta$-Carotene; aC, $\alpha$-Carotene.

Table 1. Quantification of carotenoids and chlorophylls of *Arabidopsis* T87 cell lines carrying 35S: *AHL29/SOB3*

| | Control_pGWB2 line # | | | | | | 35S: *AHL29/SOB3* line # | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | #01 | #04 | #05 | #08 | #10 | average | #01 | #03 | #05 | #08 | #16 | average | *t*-test[3] |
| Lycopene | n.d. | n.d. | n.d. | n.d. | n.d. | | n.d. | n.d. | n.d. | n.d. | n.d. | | |
| $\alpha$-Carotene | 1.09 | 0.97 | 1.07 | 1.29 | 1.00 | 1.08±0.12 | 0.75 | 0.78 | 1.07 | 0.32 | 0.50 | 0.69±0.29 | 0.03272 |
| Lutein | 20.51 | 18.48 | 23.71 | 17.92 | 19.59 | 20.04±2.28 | 12.37 | 12.09 | 17.18 | 7.44 | 7.22 | 11.26±4.12 | 0.00538 |
| $\beta$-Carotene | 5.80 | 5.33 | 6.10 | 6.64 | 5.11 | 5.8±0.61 | 3.86 | 4.08 | 5.58 | 1.81 | 2.87 | 3.64±1.41 | 0.02284 |
| Zeaxanthin | n.d. | n.d. | n.d. | n.d. | n.d. | | n.d. | n.d. | n.d. | n.d. | n.d. | | |
| Antheraxanthin[1] | 1.91 | 1.57 | 2.64 | 1.73 | 0.84 | 1.74±0.65 | 1.51 | 1.61 | 1.81 | 0.57 | 1.20 | 1.34±0.48 | 0.30712 |
| Violaxanthin[1] | 18.05 | 12.40 | 21.07 | 13.42 | 14.07 | 15.8±3.64 | 10.94 | 10.38 | 12.93 | 8.57 | 6.81 | 9.92±2.34 | 0.01961 |
| Neoxanthin[1] | 5.99 | 5.79 | 5.59 | 5.43 | 5.85 | 5.73±0.22 | 4.60 | 4.05 | 5.04 | 2.11 | 2.55 | 3.67±1.28 | 0.02165 |
| Chl a[2] | 115.02 | 109.30 | 57.41 | 112.65 | 104.48 | 99.77±24.01 | 87.79 | 97.06 | 104.54 | 19.98 | 41.32 | 70.14±37.30 | 0.17996 |
| Chl b[2] | 24.33 | 23.28 | 11.64 | 26.45 | 28.11 | 22.76±6.49 | 19.04 | 16.93 | 25.24 | 7.70 | 5.75 | 14.93±8.12 | 0.13258 |

Peaks of carotenoids were identified using authentic standards. Each peak area at 450 nm was converted to a carotenoid amount ($\mu$g pigment $g^{-1}$ fresh weight) using a standard curve of the authentic compound. The values for each individual line (single experiment) are shown and average±SD are calculated on the right.

[1] Sum of two-peak area with adjacent (difference<0.1 min) Rt and the same absorption spectra.

[2] Amounts of chlorophyll a (Chl a) and b (Chl b) were estimated ($\mu$g pigment $g^{-1}$ fresh weight) by specific absorbances of 80% acetone extract from T87 cells following the protocol reported by Lichtenthaler and Wellburn (1983).

[3] Student's *t*-test was used to determine significant differences between 35S: *AHL29/SOB3* and control vector T87 cells.

observed peaks of eight authentic standards described in Table 1, lycopene and zeaxanthin could not be detected in any of the T87 cell lines examined. For the other six carotenoids—$\alpha$-carotene, $\beta$-carotene, lutein, antheraxanthin, violaxanthin and neoxanthin—all but antheraxanthin were suppressed significantly ($P<0.05$) in the 35S: *AHL29/SOB3* transgenic lines (Table 1). In contrast, the chlorophyll content of the transgenic lines was not significantly different from that of control transgenic cells (Table 1).

The suppression of carotenoid synthesis in 35S: *AHL29/SOB3* T87 cells has several intriguing implications in light of previous studies on AT-hook motif proteins in plants. The gain- and loss-of-function experiments with *AHL29/SOB3* and *AHL27/ORE7/ESC* suggest that they function as negative regulators of hypocotyl elongation in light (Street et al. 2008). No obvious difference in cell growth of the 35S: *AHL29/SOB3* transgenic lines was seen in our experiments when grown under continuous light. Analysis of metabolites including carotenoids of 35S: *AHL29/SOB3* plants is crucially needed for comparison with T87 cultured cells. Microarray analysis of gene expression in a mutant over-expressing *AHL27/ORE7/ESC* has been carried out (Lim et al. 2007). The analysis showed that among 1096 genes that exhibited at least a two-fold change in expression compared with wild type, 615 genes were down-regulated and 481 genes were up-regulated. As our preliminary microarray experiments showed that transgenic lines over-expressing the close relative *AHL29/SOB3* exhibited down-regulation of carotenoid biosynthetic enzyme genes, we searched the microarray results of Lim et al. (2007) for those genes. Among the carotenoid biosynthetic genes, only *At3g10230* (*LCYB* for lycopene beta cyclase) was down-regulated at 0.39-fold, indicating a considerable difference from our microarray data for the T87 cells. As only data for gene expression changes over two-fold are available from their paper, however, we cannot rule out that other carotenoid biosynthetic genes are down-regulated less than two-fold in the over-expressing *AHL27/ORE7/ESC* plant. Alternatively, the over-expression of *AHL29/SOB3* might function differently in some respects from that of *AHL27/ORE7/ESC*. Further study is needed to address whether these closely related genes have redundant effects on metabolism. In the *AHL27/ORE7/ESC* over-expresser, a reduction in activity of signaling pathways involving jasmonic acid, abscisic acid, ethylene, and salicylic acid was indicated (Lim et al. 2007). Further comparative study should help elucidate the function of AT-hook motif proteins in metabolism.

## Acknowledgements

The authors thank T. Matsuura, M. Hasegawa, K. Mori, F. Fujii, K. Moriya, Y. Asami, and M. Iwata for their technical assistance. We thank the RIKEN BioResource Center (Tsukuba, Japan) for RAFL cDNA clones and *Arabidopsis* T87 cells, and Dr. T. Nakagawa (Shimane University) for his gift of pGWB2. This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO) of Japan as part of the project "Development of Fundamental Technologies for Controlling the Material Production Process of Plants".

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