Arabidopsis Mutants by Activation Tagging in which Photosynthesis Genes are Expressed in Dedifferentiated Calli

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In an effort to delineate the precise mechanisms underlying the organ-specific expression of photosynthesis genes, Arabidopsis lines homozygous for each transgene construct made with the gene for hygromycin B phosphotransferase or β-glucuronidase (GUS) placed under control of the promoter of the nuclear gene for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBCS-3B) were constructed. Furthermore, activation tagging with T-DNA possessing quadruply repeated enhancers derived from the cauliflower mosaic virus 35S promoter was applied to a transgenic line of Arabidopsis. Mutants resistant to hygromycin B during the growth of calli generated from non-green roots on callus-inducing medium resulted from the expression of hygromycin B phosphotransferase driven by the RBCS-3B promoter. Three mutant lines, ces101 to ces103 (callus expression of RBCS), were obtained from approximately 4,000 calli resistant to a selectable marker for transformation. The active transcription driven by the RBCS-3B promoter in all the calli of ces mutants was confirmed by expression of both the GUS reporter gene and endogenous RBCS-3B. Chlorophyll and carotenoids, as well as light-dependent \(O_2\) evolution, have been detected in the calli of all ces mutants. The loci where T-DNA was integrated in the ces101 line were determined by thermal asymmetric interlaced (TAIL)-PCR. The introduction of a DNA fragment harboring the gene for receptor-like kinase placed under the influence of enhancers into the parental line reproduced the phenotype of ces mutants. We have thus concluded that CES101 is a receptor-like kinase. The strategy presented in this investigation may promise to select a greater number of ces mutants.

Keywords: Activation tagging — Arabidopsis — Callus — Expression — Mutants — Photosynthesis genes.

Activation tagging utilizing enhancer sequences derived from the cauliflower mosaic virus (CaMV) 35S promoter, which were randomly integrated into chromosomes of higher plants by Agrobacterium-mediated transformation or practical use of exogenous transposons, was first successfully adapted in a study that identified a gene for auxin-independent growth (Hayashi et al. 1992). Following the success of this experimental technique, the strategy has been employed to characterize the function of dozens of genes using activation-tagged lines established independently by several research groups (Weigel et al. 2000). We designed a strategy to search mutant calli induced from roots where the expression of photosynthesis genes was initiated by activation tagging. For this purpose, we initially introduced four reporter genes placed under the control of the RBCS (gene for small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, Rubisco) and CAB (LHCB, gene for chlorophyll a/b-binding proteins) promoter. A method for screening mutants immediately after sending enhancer sequences into chromosomes of cultured cells without establishing a series of activation-tagged lines had been first applied for finding CKII, the gene for a histidine kinase homolog implicated in cytokinin signal transduction (Kakimoto 1996). We have employed this strategy in combination with reporter gene-facilitated screening of heterotrophically cultured calli of Arabidopsis.

Results

Arabidopsis lines transgenic with reporter genes driven by photosynthesis gene promoters

We focused on the expression of two nuclear genes for photosynthesis, RBCS and CAB (recent nomenclature, LHCB), which have been studied intensively. Among these gene families, it was determined that the transcriptional activity of RBCS-3B and CAB1 (LHCB1*At3) in Arabidopsis green leaves, roots and calli is under the strictest control in a green organ-specific manner (Y. Niwa, M. Shimizu, K. Goto, K. Kobayashi, M. Seki, H. Morikawa and H. Kobayashi, unpublished). The binary vectors pGA-cab-luc-rbcS-gus and pGA-cab-bar-rbcS-hph (Fig. 1A) were constructed in which the reporter genes were placed under control of the CAB1 and RBCS-3B promoters. The reporter genes consisted of the gene for firefly luciferase (luc), β-glucuronidase (uidA), the gene for phosphinothricin acetyltransferase conferring bialaphos resistance to plants (bar) and the gene for hygromycin B phosphotransferase (hph). The constructs were introduced into the roots of Arabidopsis ecotype Columbia by Agrobacterium tumefaciens GV3101-mediated transformation, followed by regeneration of the plants. From the 19 transformant lines possessing cab-luc-rbcS-gus investigated, line LG11–1 was chosen based on criteria consisting of green leaf-specific expression of uidA and luc (Table 1) and the presence of a single integrated locus (near COP9 in chromosome 4). Among the five transformant lines possessing cab-bar-rbcS-hph, line BH2-4-7 was selected due to the presence of a single integrated locus (close to nga106 in chromosome 5), and green leaf-specific expression of hph, although the roots were incapable of growing in the presence of hygromycin B. Lines LG11-1 and BH2-4-7 were crossed, self-pollinated, and one resultant line, named 2-1-6, in which both constructs were homozygously integrated, was chosen for mutant selection. When calli were induced from green leaves of line 2-1-6 harboring P_{RBCS-3B}hph, the calli were not sensitive to hygromycin B (‘leaf P_{RBCS-3B}hph’, Fig. 1B). This is possibly due to the remaining green organ-specific expression of P_{RBCS-3B}hph, resulting in the growth of calli to similar extents to those from leaves and roots of a line transgenic with P_{35S-hph} (compare with ‘leaf P_{35S-hph}’ and ‘root P_{35S-hph}’, Fig. 1B). On the other hand, roots of line 2-1-6 did not grow on callus-inducing medium (CIM) containing hygromycin B (‘root P_{35S-hph}’, Fig. 1B), indicating that these roots were suitable for selection of mutants in which RBCS is expressed in calli.

Screening for ces (callus expression of RBCS) mutants

Roots of line 2-1-6 were infected with A. tumefaciens harboring pRi35ADEn4 (Fig. 2). The T-DNA used possessed quadruply repeated enhancers derived from the CaMV 35S promoter (Hayashi et al. 1992), and the gene for acetolactate synthase which would confer resistance to sulfonylurea (Lee et al. 1988) and facilitate the selection of transformants. Roots were incubated on CIM supplemented with chlorsulfuron (a primary compound of sulfonylurea) and subsequently with hygromycin B (Fig. 2A). We obtained approximately 4,000 chlorsulfuron-resistant calli and confirmed the presence of integrated T-DNA in the calli by PCR. The T-DNA was amplified specifically from chlorsulfuron-resistant calli with the same size as that derived from pRi35ADEn4 (Fig. 3A shows some transformants), indicating that the transformation was performed successfully. Transformed calli were subjected to screening for possible hygromycin B resistance, resulting from

### Table 1

<table>
<thead>
<tr>
<th>Line</th>
<th>GUS (^a) (4-MU pmol min (^{-1}) mg protein (^{-1}))</th>
<th>Luciferase (^b) (LU mg protein (^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>Root</td>
</tr>
<tr>
<td>Wild-type</td>
<td>&lt;0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LG11–1</td>
<td>80</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Extracts were prepared from leaves and roots of 3-week-old plants grown on MS medium and calli grown on CIM.

\(^a\) GUS activity is presented in picomoles of 4-methylumbelliflorone (4-MU) per minute per microgram of protein.

\(^b\) Luciferase activity is shown in light units (LU) per milligram of protein.
Fig. 1  Constructs of binary vectors to monitor the activity of *RBCS* and *CAB* promoters (A) and photographs of calli transformed with the constructs (B). (A) Details concerning the making of constructs are described in Materials and Methods. *P*<sub>RBCS-3B</sub>, the promoter region of *RBCS*-3B; *P*<sub>CAB1</sub>, the promoter region of *CAB1*; LB, left border of T-DNA; RB, right border of T-DNA; *P*<sub>nos</sub>, the promoter of the *nopaline synthase* gene; *T*<sub>nos</sub>, the terminator of the *nopaline synthase* gene; *uidA*, the gene for β-glucuronidase; *luc*, the gene for firefly luciferase; *nptII*, the gene for neomycin phosphotransferase II; *hph*, the gene for hygromycin B phosphotransferase; and *bar*, the gene for phosphinothricin acetyltransferase conferring bialaphos resistance to plants. (B) Induction of calli from leaves and roots transgenic with *P*<sub>RBCS-hph</sub>. In an effort to determine which organ should be used for callus induction for the purpose of monitoring the activation of *P*<sub>RBCS-hph</sub> expression, line 2-1-6 harboring the *P*<sub>RBCS-hph</sub> construct, and a line possessing *P*<sub>35S-hph</sub> as a positive control, were examined. Leaves (line with *P*<sub>35S-hph</sub> and line 2-1-6 with *P*<sub>RBCS-hph</sub>) and roots (line with *P*<sub>35S-hph</sub> and line 2-1-6 with *P*<sub>RBCS-hph</sub>) were incubated on CIM medium supplemented with 20 µg ml<sup>−1</sup> hygromycin B for 4 weeks. Each panel shows a callus generated in an independent experiment.
Mutants expressing photosynthesis genes in calli

Fig. 2 Steps involved in the selection of ces calli generated from roots of Arabidopsis by activation tagging (A) and the construction of pRi35ADEn4 for the activation tagging (B). (A) The abbreviations for genes and promoters, as well as that for the plasmid, are shown in the legend for Fig. 1. (B) Details concerning the making of the constructs are described in Materials and Methods. Amp/Cb, the gene for ampicillin/carbenicillin selection; ColEl ori, the replication origin of Escherichia coli plasmid ColE1; pRiA4 ori, the replication origin of Ri plasmid A4; En, the CaMV 35S enhancer; P_CaMV35S, the CaMV 35S promoter; LB, left border of T-DNA; RB, right border of T-DNA; ALS-SU, the gene for mutated acetolactate synthase conferring resistance to sulfonylurea.
Mutants expressing photosynthesis genes in calli

Fig. 3 Confirmation of the transformation and visible phenotype of ces mutant candidates. (A) T-DNA inserts in chlorsulfuron-resistant calli. DNA was extracted from chlorsulfuron-resistant calli and PCR was carried out with the CaMV 35S promoter- and ALS-SUr-specific primers to amplify approximately 200 bp of the P35S-ALS-SUr construct. Lane 1, molecular mass makers; lane 2, amplification of pRi35ADEn4 (positive control); lane 3, amplification of pUC19 (negative control); lanes 4–7, transformant (chlorsulfuron-resistant) lines; and lane 8, parental line 2-1-6. (B) T-DNA inserts in ces mutant lines. DNA was extracted from calli and PCR was carried out with the CaMV 35S promoter- and ALS-SUr-specific primers. Lane 1, molecular mass makers; lane 2, amplification of pRi35ADEn4 (positive control); lane 3, the parental line 2-1-6; lane 4, ces101; lane 5, ces102; and lane 6, ces103. (C) Photographs of calli of ces mutant candidates, which were continuously cultured on CIM.

Fig. 4 Analysis of active transcription driven by the RBCS-3B promoter. (A) Histochemical staining reflecting GUS activity driven by PRBSC-3B-uidA in calli of ces mutant candidates. The experimental conditions employed are indicated in Materials and Methods. (B) GUS activity in ces mutant candidates. GUS activity was determined using MUG. The experimental details are given in Materials and Methods. The data are averages of three individual experiments. (C) Level of the endogenous RBCS-3B mRNA transcript in calli of ces mutant candidates. Total cellular RNA was extracted from calli. Transcript levels were determined by real-time RT–PCR as described in Materials and Methods. Transcript levels are normalized with respect to the internal standard ACT2, and are shown as the ratio of RBCS-3B/ACT2 in ces mutant candidates to that in the parental line. The data are averages of four individual experiments.
Mutants expressing photosynthesis genes in calli

Fig. 5 Characterization of photosynthetic pigments in calli of ces mutant candidates. (A) Chlorophyll fluorescence in calli of the parental line, ces101, ces102 and ces103, as well as leaves of the parental line, was observed by confocal microscopy. (B and C) Chlorophyll or carotenoid content in calli of ces mutant candidates. The data are averages of three independent experiments.

Fig. 6 Photosynthetic activity and expression of Rubisco in ces mutant candidates. (A) $O_2$ evolution and absorption in calli of the parental line and ces mutant candidates under light and dark conditions. The data are averages of four independent experiments. (B) Expression of RBCS and rbcL in terms of the protein level in calli of ces mutant candidates. A 5 µg aliquot of protein extracts from calli of the parental line, ces101, ces102 and ces103, as well as 0.1 µg of proteins from 4-week-old leaves, was electrophoresed. The experimental details are given in Materials and Methods. Rubisco was detected by immunoblotting using antibodies against spinach Rubisco. ‘L’ and ‘S’ indicate the large and small subunits of Rubisco, respectively.
the expression of P<sub>RBCS</sub>-<i>hph</i>. Throughout the continuous culture, three calli survived in the presence of chlorsulfuron and hygromycin B, the T-DNA inserts of which were confirmed by PCR (Fig. 3B). All three calli selected were pale-green, instead of the ivory yellow of their parental line (Fig. 3C). These were PCR (Fig. 3B). All three calli selected were pale-green, instead of the ivory yellow of their parental line (Fig. 3C). These were named ces101 (callus expression of RBCS-101; ‘ces’ registered in the <i>Arabidopsis</i> Gene Symbol Registry, http://www.arabidopsis.org/jsp/processor/genesymbol/symbol_main.jsp), ces102 and ces103 mutant candidates.

**GUS activity of ces mutant candidates**

Calli of ces mutants resistant to hygromycin B were analyzed for P<sub>RBCS</sub>-<i>uidA</i> expression by staining with X-glue. Calli of the parental line 2-1-6 in which <i>RBCS-3B</i> was not expressed did not stain (Fig. 4A), although the leaves in which <i>RBCS-3B</i> was strongly expressed stained blue (data not shown). Calli of ces mutants were stained blue (Fig. 4A), indicating that <i>uidA</i> was strongly expressed under control of the <i>RBCS-3B</i> promoter. GUS activity was also determined using 4-methylumbelliferylglucuronide (MUG). While no activity was detected in calli of the parental line, the activity was high in the ces mutant calli (Fig. 4B). The overall results indicate that the <i>RBCS-3B</i> promoter was active in ces mutant calli and resulted in the expression of <i>hph</i> and <i>uidA</i>.

**Expression of endogenous RBCS-3B in ces mutant candidates**

The level of endogenous <i>RBCS-3B</i> mRNA transcript was determined by real-time reverse transcription–PCR (RT–PCR). Total RNA, extracted from the calli of ces mutants and the parental line, was subjected to cDNA synthesis using reverse transcriptase. The gene for actin 2 (ACT2) was employed as an internal normalization standard. The level of <i>RBCS-3B</i> mRNA transcript was approximately 700 times higher in ces101 calli, 30 times higher in ces102 calli and 500 times higher in ces103 calli than that in calli of the parental line (Fig. 4C).

**Table 2 Photosynthesis genes expressed specifically in ces101 calli**

<table>
<thead>
<tr>
<th>MIPS code</th>
<th>Gene product</th>
<th>ces101/parental line</th>
</tr>
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<tbody>
<tr>
<td>At3g26650</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase A subunit (GapA)</td>
<td>40.0</td>
</tr>
<tr>
<td>At5g54270</td>
<td>Chlorophyll a/b-binding protein</td>
<td>35.3</td>
</tr>
<tr>
<td>At1g10960</td>
<td>Ferredoxin precursor isolog</td>
<td>16.3</td>
</tr>
<tr>
<td>At1g76100</td>
<td>Plastocyanin</td>
<td>14.3</td>
</tr>
<tr>
<td>At5g66570</td>
<td>33 kDa polypeptide of oxygen-evolving complex (OEC) in PSII</td>
<td>14.3</td>
</tr>
<tr>
<td>At5g64040</td>
<td>PSI reaction center subunit psaN precursor (PSI-N)</td>
<td>14.2</td>
</tr>
<tr>
<td>At1g61520</td>
<td>PSI type III chlorophyll a/b-binding protein, putative</td>
<td>13.6</td>
</tr>
<tr>
<td>At1g79040</td>
<td>Photosystem II polypeptide, putative</td>
<td>12.8</td>
</tr>
<tr>
<td>At3g47470</td>
<td>Chlorophyll a/b-binding protein 4 precursor</td>
<td>12.0</td>
</tr>
<tr>
<td>At3g54890</td>
<td>Chlorophyll a/b-binding protein</td>
<td>10.0</td>
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The Agilent oligo-DNA microarray (14,880 genes) was employed.

**Photosynthetic activity of ces mutant calli**

Chl fluorescence was not detected in calli of the parental line by confocal microscopy (Fig. 5A). However, it was emitted in cells of ces101, ces102 and ces103 calli, where the sizes of compartments emitting the fluorescence were smaller than those in leaves of the parental line. The fluorescent compartments seem to be held against the cell walls in calli of all ces mutant lines, where central vacuoles would develop well. Chl fluorescence was not detected in calli of the parental line by confocal microscopy (Fig. 5A). However, it was emitted in cells of ces101, ces102 and ces103 calli, where the sizes of compartments emitting the fluorescence were smaller than those in leaves of the parental line. The fluorescent compartments seem to be held against the cell walls in calli of all ces mutant lines, where central vacuoles would develop well. Chl <i>a</i> and <i>b</i> were undetectable in calli of the parental line, whereas those of ces mutants were high (Fig. 5B) but lower than those in green leaves (data not shown). Carotenoids also accumulated more in calli of ces mutants than in those in the parental line (Fig. 5C). The photosynthetic activity as light-dependent O<sub>2</sub> evolution was detected in the calli of all ces mutants, when O<sub>2</sub> absorption by respiration was observed in the calli of the parental line in the light and in all the calli in the dark (Fig. 6A). The expression of Rubisco in ces mutant calli was examined at the protein level by immunoblotting (Fig. 6B), when the size of the small subunit of Rubisco, the <i>RBCS</i> gene product, is equivalent to that in leaves of the parental line. Therefore, the small subunit molecules seem to be properly transported into chloroplasts in ces mutant calli. Total cellular RNA was extracted from calli of ces101 and the parental line, and subjected to oligo-DNA microarray (14,880 genes, Agilent Technologies). The levels of transcripts for a dozen genes related to photosynthesis were higher in calli of ces101 than in those in the parental line (Table 2).

**Identification of genes governing the phenotype**

The integration of one copy of T-DNA per genome was detected in the ces101 line by Southern hybridization (data not shown), and thermal asymmetric interlaced (TAIL)-PCR (Liu et al. 1995) revealed its location at approximately 6 Mb from the top of chromosome 3, the region covered by P1 clone MSL1, with a 70 bp deletion of chromosome DNA at the right border of integrated T-DNA. There were genes for receptor-like kinase (RLK) (At3g16030), unknown protein (At3g16040) and...
Mutants expressing photosynthesis genes in calli

Fig. 7 Identification of CES101. (A) Schematic diagram of the location and structure of genes surrounding the inserted T-DNA. The intron–exon structure of the coding strand is shown with black rectangles; introns are shown with thin black lines between exons. (B) Photographs of calli where causal gene candidates were ectopically expressed. A DNA fragment harboring either RLK or kinesin was combined with quadruply repeated enhancer sequences and integrated into the binary vector pBCH1 to generate pBEn4R or pBEn4K as described in Materials and Methods. Calli induced from roots of the parental line 2-1-6 were subjected to Agrobacterium-mediated transformation with pBEn4R or pBEn4K. Calli were cultured on CIM for 4 weeks. Each panel shows an independent transformant callus, with pBEn4R1 to pBEn4R3 or with pBEn4K1 to pBEn4K3. (C) Transcript levels of the gene for RLK located near the integrated T-DNA in ces101 calli. Real-time RT–PCR was performed with total RNA extracted from calli of the parental line, ces101, and transformant callus with pBEn4R and pBEn4K. (D) Transcript levels of the RLK gene in the different organs. Total cellular RNA was extracted from calli, 5-week-old leaves, 3-week-old roots and flowers of the wild-type line (Columbia). Transcript levels were determined by real-time RT–PCR using a LightCycler (Roche). Transcript levels are normalized with the internal standard ACT2, and indicated as their ratios to levels in calli of the wild-type line.
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Discussion

The screening and examination of Arabidopsis photomorphogenetic mutants such as det (de-etiolated) (Chory et al. 1989, Pepper et al. 1994), fus (fusca) (Castle and Meinke 1994) and cop (constitutively photomorphogenic) (Deng et al. 1991, Deng et al. 1992, Wei et al. 1994) added credence to the notion that factors which can suppress the expression of photosynthesis genes exist, and that these participate in non-green morphogenesis. On the other hand, cue (CAB underexpressed) mutants in which positive regulators of light-controlled CAB expression are disordered have been reported (López-Juez et al. 2001) to generate pBen4R and pBen4K, respectively. Calli induced from roots of the parental line 2-1-6 were transformed with the constructs. Calli generated from independent transformants with pBen4R were pale-green, whereas calli transformed with pBen4K were ivory yellow (Fig. 7B). When RNA prepared from the calli transformed with either pBen4R or pBen4K was analyzed by real-time RT–PCR, significantly enhanced levels of transcripts for RLK were found only in calli transgenic with pBen4Rs but not pBen4Ks (Fig. 7C). The transcript levels of RBCS-3B were also elevated, in agreement with the extent of expression of RLK (data not shown). Therefore, the phenotype of ces101 is ascribed to the expression of the gene for RLK (referred to as CES101 hereafter). The transcript levels of CES101 in each organ were determined by real-time RT–PCR, resulting in expression of CES101 predominantly in leaves, whereas it was low in calli, roots and flowers (Fig. 7D).

We have determined the locus of T-DNA insertion in the ces101 line by TAIL-PCR, and identified the gene for RLK to be CES101 whose expression causes the phenotype. Enhanced expression of CES101 resulted in RBCS transcription in transformant calli, indicating that CES101 as an RLK may regulate the expression of photosynthesis genes. The RLK family is composed of 610 members (Shiu and Bleecker 1999), which is reported to participate in various signal transductions. The first category includes RLKs that control plant growth and development, for example ERECTA in determining organ shape (Torii et al. 1996), CLAVATA1 in controlling differentiation in shoot meristems (Clark et al. 1996), BRI1 identified to be a brassinosteroid receptor capable of combining with brassinolide, subsequently in transmitting brassinosteroid signals (Kinoshita et al. 2005), maize CRINKLY4 in governing cell morphogenesis and differentiation (Becraft et al. 1996), and Brassica rapa SRK in regulating self-incompatibility (Takasaki et al. 2000). The second category includes RLKs involved in plant–microbe interactions and defense responses (Shiu et al. 2004). CES101 possesses putative domains of S-locus glycoprotein (SLG) and Ser/Thr protein kinase, and has been classified into the RLK subfamily SD-1b (Shiu et al. 2004) to which ARK1, the Arabidopsis counterpart of SRK involved in self-incompatibility through interaction with S-locus protein 11 (SP11), belongs (Takayama et al. 2001). ARK1 exhibiting the highest homology to CES101 among RLKs (Shiu and Bleecker 2001), has been proved to be associated with kinase activity (Tobias and Nasrallah 1996). Therefore, CES101 may have its activity. The discovery of participation of CES101 has indeed added an additional example to the roles of the RLK family.

We have presented the evidence for transcriptional regulation of plastid photosynthesis genes in a green organ-specific manner (Isono et al. 1997a), and studied nucleus-encoded σ factors of plastid RNA polymerase involved in the regulation (Isono et al. 1997b). Nucleus-regulated plastid gene expression and plastid to nucleus signaling (Rodermel 2001) are of great interest. The analysis of the ces series of mutants, three of

ethylene-responsive protein (At3g16050) near to the left border of T-DNA, as well as for kinesin (At3g16060) close to the right border (Fig. 7A). The transcript level of RLK was enhanced 2-fold in calli of ces101 compared with the parental line, as revealed by real-time RT–PCR (Fig. 7C), while transcript levels of unknown protein, ethylene-responsive protein and kinesin did not increase (data not shown). Therefore, it is supposed that the expression of RLK may contribute the phenotype to ces101 calli. To confirm whether the ectopic expression of RLK would reproduce the phenotype of ces101, a DNA fragment harboring either RLK or kinesin was picked up from P1 clone MSL1, combined with quadruply repeated enhancer sequences and introduced into the binary vector pBCH1 (Ito et al. 2001) to generate pBen4R and pBen4K, respectively. Calli induced from roots of the parental line 2-1-6 were transformed with the constructs. Calli generated from independent transformants with pBen4R were pale-green, whereas calli transformed with pBen4K were ivory yellow (Fig. 7B). When RNA prepared from the calli transformed with either pBen4R or pBen4K was analyzed by real-time RT–PCR, significantly enhanced levels of transcripts for RLK were found only in calli transgenic with pBen4Rs but not pBen4Ks (Fig. 7C). The transcript levels of RBCS-3B were also elevated, in agreement with the extent of expression of RLK (data not shown). Therefore, the phenotype of ces101 is ascribed to the expression of the gene for RLK (referred to as CES101 hereafter). The transcript levels of CES101 in each organ were determined by real-time RT–PCR, resulting in expression of CES101 predominantly in leaves, whereas it was low in calli, roots and flowers (Fig. 7D).
which we describe here and we may obtain more in continuous screening, could provide clues to discovering the mechanisms underlying the intracellular signaling for chloroplast biogenesis.

Materials and Methods

Constructs for reporter genes

DNA fragments covering the promoter region (approximately 1.5 kbp each) of \( CABI \) (LHCB1*At3) for the chlorophyll a/b-binding proteins, or \( RBCS-3B \) (Niwa et al. 1997) for the small subunit of Rubisco from \( Arabidopsis \) (Columbia) were positioned so as to drive the expression of the \( luc \) or \( uidA \) genes, respectively. These constructs were combined to make \( P_{cab-luc} \)-\( P_{rbcS-uidA} \) (\( luc \) was derived from \( pDO432 \), Ow et al. 1986; and \( uidA \) was derived from \( parAs-GUS \), Niwa et al. 1994). The same promoter regions were also placed ahead of the \( bar \) and \( hph \) genes, to generate \( P_{cab-bar} \)-\( P_{rbcS-hph} \) (\( bar \) was derived from \( pARK22 \), Sawasaki et al. 1995; and \( hph \) was derived from \( pDH25 \), Cullen et al. 1987). The \( HindIII-NotI \) fragment harboring \( P_{cab-luc-Tnos} \) and the \( NotI-KpnI \) fragment harboring \( P_{rbcS-uidA-Tnos} \) were ligated to the \( HindIII- \) and \( KpnI \)-digested binary vector \( pGAomega \) [derived from \( pGA469 \) (An 1987) and combined with \( pUC18 \) at the \( EcoRI \) site], resulting in the generation of a plasmid named \( pGA-cab-luc-rbcS-gus \) (Fig. 1A). The same restriction enzyme sites were used to construct \( pGA-cab-bar-rbcS-hph \) in the binary vector (Fig. 1A).

Genetic transformation

Transformation was carried out with \( A. \) \( tumefaciens \) GV3101 (Konez et al. 1989). \( Arabidopsis \) transformant lines homozygous with a single locus of each of the aforementioned joined constructs were crossed and self-pollinated. Line 2-1-6, in which both constructs were homozygously integrated in a different chromosome (chromosomes 4 and 5), was chosen for mutant selection.

Activation tagging and mutant selection

Seeds were surface sterilized, sown in plastic Petri dishes containing solidified Murashige–Skoog (MS) medium with gellan gum (San-Ei Gen F.F.I., Inc., Toyonaka, Japan), and vernalized for 2 d at 4°C (Tsugane et al. 1999). Following 7 d of incubation in growth chambers (at 20°C, continuous white fluorescent light), 15–20 seedlings were transferred to flasks containing liquid MS medium and subsequently grown with shaking at 80 rpm for 2 weeks. Thereafter, cultured roots were detached from green tissues (leaves and stems), cut into 3–6 mm segments, transferred to CIM (MS medium supplemented with 0.5 \( \mu \)g ml\(^{-1}\) 2,4-D and 50 \( \mu \)g ml\(^{-1}\) kinetin; Valkvens et al. 1988), and incubated in growth chambers for 5 d.

The binary vector for activation tagging, \( p\) \( Rl35ADEn4 \) (Fig. 2B), was constructed through the ligation of three DNA fragments. The replication origin region of \( p\) \( RL4A \) and the left border (\( LB \)) were derived from \( pBU2G2R \) (Niwa et al. 2003), while the quadruple enhancer of the \( CaMV \) 35S promoter and right border (\( RB \)) were derived from \( pPVCICEn4HPT \) (Hayashi et al. 1992). The sulfonylurea-resistant acetolactate synthase gene cassette with P196A and W573L mutations was derived from the plasmid \( pUC35AD \) (kindly provided by Barbara J. Mazur, E.I. du Pont de Nemours & Co., Wilmington, DE, USA; Lee et al. 1988). The roots were infected with \( A. \) \( tumefaciens \) GV3101 harboring \( p\) \( Rl35ADEn4 \). Following co-culturing, the roots were washed with liquid CIM supplemented with 0.1 mg ml\(^{-1}\) cesfotaxime. The roots were then cultured on CIM in the presence of 0.2 mg ml\(^{-1}\) vancomycin and 0.1 mg ml\(^{-1}\) chlorsulfuron for transformant selection over a 3 week period. Transformed calli were then transferred to CIM supplemented with 0.2 mg ml\(^{-1}\) vancomycin, 0.1 mg ml\(^{-1}\) cesfotaxime, 0.1 \( \mu \)g ml\(^{-1}\) chlorsulfuron and 20 \( \mu \)g ml\(^{-1}\) hygromycin. B. Mutant candidates were repeatedly selected on the medium.

Identification of T-DNA inserts by PCR

Genomic DNA was extracted using Isoplant (NIPPON GENE CO., LTD., Toyama, Japan) or as described (Weigel and Glazebrook 2002) from calli that grew on CIM containing 0.1 mg ml\(^{-1}\) chlorsulfuron. This DNA was then subjected to PCR in an effort to amplify approximately 200 bp of \( P_{cab-luc} \)-\( ALS-SE \) using the primers 35Smimil-\( fd \) (Niwa et al. 1999) and \( ALS22-rv \) (Table 3), followed by agarose gel electrophoresis with 3% (w/v) agarose21 (NIPPON GENE CO., LTD.).

Table 3  List of PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>35Smimil-fd</td>
<td>GCAAGACCCCTCCTCTATATAAGG</td>
<td>Niwa et al. (1999)</td>
</tr>
<tr>
<td>ALS22-rv</td>
<td>TCTAGGGAGAGGGGGTTGGAAGTTT</td>
<td>This study</td>
</tr>
<tr>
<td>LB1</td>
<td>CCTTATAGAGGAAGGGTGTTGC</td>
<td>This study</td>
</tr>
<tr>
<td>LB2</td>
<td>TGGGATTTGCGTCTACCTTACAG</td>
<td>This study</td>
</tr>
<tr>
<td>LB3</td>
<td>TTTGAGATTGCCCGATTGCCCTCA</td>
<td>This study</td>
</tr>
<tr>
<td>AD2</td>
<td>NGTCGA(G/C)(A/T)GANA(A/T)TGAA</td>
<td>Liu et al. (1995)</td>
</tr>
<tr>
<td>AD3</td>
<td>(A/T)TGTNAG(A/T)ANCANAGA</td>
<td>Liu et al. (1995)</td>
</tr>
<tr>
<td>AD2-2’</td>
<td>GTNCGA(G/C)(A/T)CANATGTTTT</td>
<td>Liu et al. (2005)</td>
</tr>
<tr>
<td>CES101-rv</td>
<td>AACATCTCAGTTGATGAGCA</td>
<td>This study</td>
</tr>
<tr>
<td>RB3</td>
<td>GGATTGATGTATCTAGATCCGG</td>
<td>This study</td>
</tr>
<tr>
<td>RBCS-le-fd</td>
<td>CGCGCAACAAAGTTGATCCCTTG</td>
<td>This study</td>
</tr>
<tr>
<td>RBCS-3B-s-rv</td>
<td>AATGAGCAGAGATAATTCATAAGATG</td>
<td>This study</td>
</tr>
<tr>
<td>ACT2-fd</td>
<td>GAAGATTAAGGGTCTGTGCAACCACCTG</td>
<td>This study</td>
</tr>
<tr>
<td>ACT2-rv2</td>
<td>ATCTCTGAGCTGTCCTATATCTC</td>
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</tr>
<tr>
<td>RLK-fd</td>
<td>AATCCTATAGTCCATGTTGGAATC</td>
<td>This study</td>
</tr>
<tr>
<td>RLK-rv</td>
<td>CTTCGAAATTCCTAGTGCTATTTGGG</td>
<td>This study</td>
</tr>
</tbody>
</table>
Mutants expressing photosynthesis genes in calli

GUS assays

Candidate calli were analyzed for RBCS promoter activity by staining with X-gluc. Staining solution included 0.5 mg ml⁻¹ X-gluc, 0.3% (v/v) Triton X-100, 20% (v/v) methanol, 0.5 mM K-ferricyanide and 0.5 mM K-ferrocyanide as catalysts in 0.1 M Na-phosphate buffer (pH 7.0). Mutant and parental line leaves and calli were stained overnight with the solution at 37°C in the dark.

GUS activity was determined using fluorometric MUG. Calli were ground using a mortar and pestle in the presence of extraction buffer consisting of 0.1 M Na-phosphate (pH 7.5) and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 21,500g for 1 min, and the supernatant was centrifuged again. The protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Portions (2 µl) of the supernatant were added to reaction solution (0.5 ml finally) consisting of 1 mM MUG, 50 mM Na₂PO₄ (pH 7.0), 10 mM EDTA, 0.1% (v/v) Na-laurylsarcosine, 0.1% (v/v) Triton X-100 and 20% (v/v) methanol, and incubated at 37°C. Following incubation for 5, 10, 15 and 20 min, 100 µl of reaction mixture was taken and added to 0.9 ml of 0.2 M Na₂CO₃. The fluorescence intensity was determined using a Microtiter Plate Reader (Hitachi, Tokyo, Japan) with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. GUS activity is presented as 4-MU pmoles min⁻¹ µg protein⁻¹.

Real-time RT–PCR

Total cellular RNA was extracted using Isogen (NIPPON GENE CO., LTD.). Following the cleavage of genomic DNA by RNase-free DNase I (TAKARA SHUZO CO. LTD., Otsu, Japan), the RNA was subjected to cDNA synthesis using the First Strand cDNA Synthesis Kit (Takara, Shiga, Japan). The RNA was digested with RNase-free DNase (TAKARA SHUZO CO., LTD.). Following the cleavage of genomic DNA by RNasefree DNase I (TAKARA SHUZO CO. LTD.), the RNA was further purified using an RNase Mini Kit (Qiagen, Hilden, Germany). The total cellular RNA (20 µg) was used for cDNA synthesis, labeling and subsequent hybridization by an Arabidopsis 1 Oligo Microarray Kit (Agilent Technologies, Palo Alto, CA, USA). Microarray analysis was repeated in two independent experiments. The results are shown as averages of the data whose P-values were ≤0.01 in two independent experiments.

Thermal asymmetric interlaced (TAIL)-PCR

Total DNA was extracted from the mutant calli and subjected to TAIL-PCR (Liu et al. 1995, Liu et al. 2005). Primers specific to the T-DNA left borders were LB1, LB2 and LB3 (Table 3). The degenerate primers used were AD2, AD3, and AD2–2 (Table 3). After TAIL-PCR, fragments were separated by gel electrophoresis and purified from gels. Purified fragments were directly sequenced. Since it was difficult to amplify the region of the right border, the CES101-rv primer (Table 3) was designed in a region harbored by P1 clone MSL1, and was amplified with a primer specific to right border, RB3 (Table 3).

Construction of binary vectors for identifying causal genes

P1 clone MSL1 was digested with XbaI and separated by agarose gel electrophoresis. The 6.8 kb fragment containing the RLK gene and the 8.7 kb fragment possessing the kinesin gene were retrieved from gels. Two fragments, respectively, were cloned into pGEM-3zf (Promega Corporation, Madison, WI, USA) and digested with XbaI to generate pRE and pK harboring genes for RLK and kinesin.

For pBEn4R, the DNA fragment harboring quadruply repeated enhancers between SpeI and the outermost EcoRV sites in pRI35ADεn4 was cloned into pGEM-SZ1 (Promega Corporation) to make pEn4. pRE was digested with SphI and KpnI, and pEN4 was digested with SphI and SpeI. These two fragments were retrieved from gels and co-ligated with the SpeI–KpnI backbone cloned into pBCH1 (Ito et al. 2001) to make pBEn4RE, which was further digested with SpeI, and the resultant fragment containing enhancer sequences and the gene for RLK was cloned into pBCH1 digested with SpeI to generate pBEn4R.

For pBEn4K, the fragment between AraI and the outermost XhoI sites in the insert of pK, and that cut out with AraII and SpeI from pEn4 were co-ligated with the SpeI–XhoI backbone of pBCH1.

Acknowledgments

The authors are grateful to Stephen H. Howell, Hiroyuki Anzai, Richard Walden, Yukihiro Ito and Barbara J. Mazur for kindly providing the pDO432, pARK22, pPCVICεn4HPT, pBCH1 and pUC35AD proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane, Hybrid-P (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with Block Ace (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) and then incubated in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 and primary and secondary antibodies which were diluted 1:50,000 and 1:10,000, respectively. The primary antibodies represented the IgG fraction of rabbit antibodies against spinach Rubisco (Ngernprasirtsiri et al. 1998), while the secondary antibodies were goat anti-rabbit IgGs conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA, USA). Signals were detected using enzyme catalysed fluorescence (ECF) as the substrate (Amersham Biosciences).
plasmids, respectively. We express sincere thanks to Mikio Nishimura and Makoto Hayashi for their help in scanning Aligent oligo-DNA microarray chips. We wish to thank ABRC (Arabidopsis Biological Resource Center, USA) for the p35S-lhph Arabidopsis line. This research was supported by Grants-in-Aid and the COE Program in the 21st Century from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT, Mombukagakusho), by the Project Research Grant of the Salt Science Research Foundation, and by the Goto Research Grant from University of Shizuoka.

References


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