The SCABRA3 Nuclear Gene Encodes the Plastid RpoTp RNA Polymerase, Which Is Required for Chloroplast Biogenesis and Mesophyll Cell Proliferation in Arabidopsis1[W]

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In many plant species, a subset of the genes of the chloroplast genome is transcribed by RpoTp, a nuclear-encoded plastid-targeted RNA polymerase. Here, we describe the positional cloning of the SCABRA3 (SCA3) gene, which was found to encode RpoTp in Arabidopsis (Arabidopsis thaliana). We studied one weak (sca3-1) and two strong (sca3-2 and sca3-3) alleles of the SCA3 gene, the latter two showing severely impaired plant growth and reduced pigmentation of the cotyledons, leaves, stem, and sepals, all of which were pale green. The leaf surface was extremely crumpled in the sca3 mutants, although epidermal cell size and morphology were not perturbed, whereas the mesophyll cells were less densely packed and more irregular in shape than in the wild type. A significant reduction in the size, morphology, and number of chloroplasts was observed in homozygous sca3-2 individuals whose photoautotrophic growth was consequently perturbed. Microarray analysis showed that several hundred nuclear genes were differentially expressed in sca3-2 and the wild type, about one-fourth of which encoded chloroplast-targeted proteins. Quantitative reverse transcription-PCR analyses showed that the sca3-2 mutation alters the expression of the rpoB, rpoC1, clpP, and accD plastid genes and the SCA3 paralogs RpoTmp and RpoTmp, which respectively encode nuclear-encoded mitochondrion or dually targeted RNA polymerases. Double-mutant analysis indicated that RpoTmp and SCA3 play redundant functions in plant development. Our findings support a role for plastids in leaf morphogenesis and indicate that RpoTp is required for mesophyll cell proliferation.

Leaf development is regulated by environmental signals and endogenous cues, together with their cross talk, by means of genetic networks that modulate cell division, expansion, and differentiation. Light is one of the most important signals controlling leaf development because it triggers differentiation of non-photosynthetic proplastids into fully functional photosynthetic chloroplasts (López-Juez and Pyke, 2005; Waters and Pyke, 2005). The identification and characterization in different plant species of mutants with aberrant leaf anatomy, affected in nuclear genes encoding proteins with chloroplast-related functions, highlights the important role of chloroplast biogenesis in leaf morphogenesis and overall plant development. Thus, the Antirrhinum majus differentiation and greening (dag) mutant (Sommer et al., 1985; Chatterjee et al., 1996), the Arabidopsis (Arabidopsis thaliana) mutants pale cress (pac; Reiter et al., 1994; Grevelding et al., 1996), DAG-like1 (dai1; Babiychuk et al., 1997; despite the sequence similarity between DAL1 and DAG proteins, the corresponding genes are not orthologous [Bisanz et al., 2003]), cloroplastos alterados1 (cla1; Mandel et al., 1996), yellow variegated1 and 2 (var1 and var2; Chen et al., 2000; Takechi et al., 2000; Sakamoto et al., 2002), immutans (im; Wetzel et al., 1994; Carol et al., 1999; Wu et al., 1999), CAB underexpressed1 (cue1; Li et al., 1995; Streathfield et al., 1999), and phosphatidylglycerolphosphate synthetase1 (pgp1; Hagio et al., 2002) and the tomato (Lycopersicon esculentum) defective chloroplast and leaves-mutable (dcl; Keddie et al., 1996) mutants are albinos or display pale or whitish sectors as a consequence of damaged nuclear genes encoding chloroplast proteins. The fact that the structure of the internal leaf tissues is altered in all of these mutants suggests a connection between chloroplast and mesophyll development (Chatterjee et al., 1996; Keddie et al., 1996) and supports the hypothesis that leaf morphogenesis is controlled by plastid-to-nucleus signaling (Pyke et al., 2000; Rodermel, 2001).

The effect on leaf and whole-plant development of mutations in genes encoding proteins of the transcriptional machinery of plastids is beginning to emerge.

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The transcription of chloroplast genes is carried out by two types of plastid RNA polymerases (RNAPs), which are plastid encoded (PEP) or nuclear encoded (NEP; for review, see Hess and Börner, 1999). PEPs are multisubunit polymerases that require σ-like factors encoded by the nucleus, similar to the RNAPs found in eubacteria such as Escherichia coli (Igloi and Kössel, 1992; Allison et al., 1996; Allison, 2000), whereas NEPs belong to the family of bacteriophage-type T3 and T7 RNAPs (RpoTs; Lerbs-Mache, 1993). It has been proposed that transcription of the photosynthetic genes harbored by the plastid genome is preferentially driven by PEP, whereas NEP transcribes genes encoding components of the plastid genetic system, such as those of the translational apparatus and PEP core subunits (Allison et al., 1996; Hajdukiewicz et al., 1997; Magee and Kavanagh, 2002). However, other studies indicate an overlap of PEP and NEP activities (Krause et al., 2000; Legen et al., 2002).

Two dually targeted organelle NEPs have been identified in the moss Physcomitrella patens (Richter et al., 2002). Monocotyledonous plants possess one NEP specific to mitochondria and another specific to chloroplasts (Ikeda and Gray, 1999; Emanuel et al., 2004; Kusumi et al., 2004). Three organelle NEPs have been identified in Arabidopsis and other dicotyledonous species: RpoTm is targeted to the mitochondria, RpoTp to the chloroplasts, and RpoTmp to both of these organelles (Hedtke et al., 1997, 1999, 2000). Orthologs of the RpoT genes of Arabidopsis have been cloned in P. patens (Richter et al., 2002), Chenopodium album (Weihe et al., 1997), Hordeum vulgare (Emmanuel et al., 2004), tobacco (Nicotiana tabacum), Nicotiana sylvestris, Nicotiana tomentosiformis (Kobayashi et al., 2001; Hedtke et al., 2002), Oryza sativa (Kusumi et al., 2004), Triticum aestivum (Ikeda and Gray, 1999), and Zea mays (Chang et al., 1999). It is assumed that plastid-type RpoT genes derive from an original mitochondrion-type RpoT gene by gene duplication (Hess and Börner, 1999; Kabeya et al., 2002). Mutational analyses on the role of RpoT genes in plant development are scarce, one of the few exceptions being the recent isolation and characterization of the rpoT1-2 mutant of Arabidopsis (Baba et al., 2004), which exhibits altered leaf morphology and a general reduction in growth, consistent with the assumed relationship between chloroplast and leaf development.

To gain insight into the genetic and molecular mechanisms involved in the control of leaf ontogeny, we performed a large-scale screening for ethyl methanesulfonate (EMS)-induced mutants with abnormal leaves in Arabidopsis (Berná et al., 1999). We report here our studies of one of these EMS-induced mutations, scabra3-1 (sca3-1), together with that of two additional insertional alleles of the SCA3 gene, which was positionally cloned and found to encode the RpoTp protein. Loss of function of SCA3 affected plastid and nuclear gene expression in Arabidopsis, severely perturbing chloroplast development and leaf morphology.

RESULTS

Positional Cloning of the SCA3 Gene

In a large-scale screening for EMS-induced mutants with altered leaf morphology, we isolated seven mutants of Arabidopsis displaying rounded and pale-green leaves, protruding leaf laminae, and irregular leaf margins (Fig. 1). The corresponding mutations were found to be recessive and completely penetrant, with only small variations in expressivity. They were grouped in a phenotypic class that we named Scabra (Sca) and found to fall into five complementation groups (SCA1 to SCA5; Berná et al., 1999). The SCA genes have been low-resolution mapped (Robles and Micol, 2001).

To positionally clone the SCA3 gene, we performed outcrosses of sca3-1/sca3-1 plants in a Landsberg erecta (Ler) genetic background to Columbia-0 (Col-0). Linkage analysis of the F2-mapping populations obtained allowed us to delimit a candidate region of 70 kb, containing 16 annotated genes (Fig. 2A). We then searched for publicly available lines bearing T-DNA insertions within the candidate interval and found two (N593884 and N567191) that displayed a recessive phenotype more extreme than that of the sca3-1 mutant (Fig. 1). T-DNA insertions were confirmed at nucleotide positions 1,894 (numbering from the predicted translation initiation codon) in N593884 and 5,194 in N567191, disrupting the third intron and eighteenth exon, respectively, of the At2g24120 transcription unit (Fig. 2B), as annotated at the SIGnAL Web site (http://signal.salk.edu). Allelism tests were performed by crossing sca3-1/sca3-1 plants to phenotypically mutant N593884 and N567191 individuals, and the F1 progeny were phenotypically mutant. Because these results indicated that the gene perturbed in the EMS-derived sca3-1 mutant is At2g24120, we consequently named the T-DNA alleles carried by the N593884 and N567191 lines as sca3-2 and sca3-3, respectively. Given that the phenotypes of the latter two mutants were indistinguishable, the sca3-2 allele was chosen for further study.

Molecular Characterization of sca3 Alleles

We sequenced the At2g24120 transcription unit in the sca3-1 mutant and its wild-type Ler and found a G → T transversion at position 4,856, affecting the splicing donor site of the sixteenth exon (Fig. 2, B and C). To examine the effect of the sca3 mutations on SCA3 gene expression, total RNA was extracted from 3-week-old sca3/sca3, Ler, and Col-0 plants and subjected to reverse transcription (RT)-PCR, and the amplification products were sequenced. Using primers that hybridize upstream of both T-DNA insertions (At2g24120F7 and R7; Supplemental Table I; Fig. 2, B and E), a single band of the expected size (548 bp) was detected in all cases, corresponding to similar amounts of RT-PCR products from sca3-2/sca3-2, sca3-3/sca3-3, and Col-0 plants, whereas slightly fewer amplification products
were obtained from sca3-1/sca3-1 than from Ler plants (Fig. 2E). Similar results were obtained using different primer set combinations.

Two different SCA3 cDNAs were found in the sca3-1 mutant (see “Materials and Methods;” Supplemental Fig. 1), one of which carries only a G → T change, and the other lacks 8 bp (Fig. 2C). As a consequence, translation of sca3-1 mRNAs would produce two proteins, one of which has no amino acid difference with the wild type and the other lacks 109 amino acids of its C-terminal region due to a frameshift and a premature stop codon (Fig. 2D). Primers flanking the T-DNA insertions (F2 and R3 for sca3-2; F4 and R9 for sca3-3) yielded amplification products only from Col-0, but not from sca3-2 and sca3-3 cDNA or genomic DNA (Fig. 2F). Sequencing of additional amplification products (obtained using the F2 and LBa1 primers; Fig. 2, B and F) indicated that a chimeric transcript is obtained in sca3-2, which includes 1,667 nucleotides of SCA3 mRNA and at least 272 nucleotides of the left border of the T-DNA insert. Translation of this chimeric transcript would produce a truncated protein of 389 amino acids, lacking 604 residues of the C-terminal part of the wild-type protein (Fig. 2G). We did not characterize sca3-3 mRNA, which was assumed to encode a protein lacking 50 amino acids in its C-terminal region and probably including some divergent amino acids, translated from the T-DNA insert.

Effects of sca3 Mutations on Expression of RpoT Nuclear Genes

The predicted product of the SCA3 gene is a protein of 993 amino acids with a molecular mass of 112.6 kD (http://www.arabidopsis.org/index.jsp) corresponding to the T7 phage-type RNAP, RpoTp, which is targeted exclusively to plastids (Hedtke et al., 1997, 1999). RpoTp is a single-subunit RNAP that contains 11 well-conserved domains (Fig. 3, I–XI; McAllister and Raskin, 1993). The frameshift caused by the sca3-1 mutation would remove the X and XI domains, including the so-called C-motif, which is part of the catalytic site of T7 RNAPs. The sca3-2 mutation would remove the III to XI domains (Fig. 3), which suggests that it abolishes RpoTp function.

Using quantitative RT (qRT)-PCR, we studied the expression of the RpoTm, RpoTmp, and SCA3 (RpoTp) genes of Arabidopsis in 4-d-old seedlings (showing...
only expanded cotyledons), 12-d-old seedlings (showing expanded cotyledons and four expanding leaves), 3-week-old rosettes (see Fig. 1, G and H), and roots (Table I). The three genes were found to be expressed in all the organs and stages analyzed in wild-type and sca3-2/sca3-2 plants. In Col-0, RpoTm reached the highest level of expression in 12-d-old seedlings, where it was 1.9-, 1.5-, and 2.1-fold higher than in 4-d-old seedlings, rosettes, and roots, respectively. On the contrary, RpoTmp and SCA3 were predominantly expressed in rosettes, especially SCA3, whose transcripts accumulated 3.3-, 2.5-, and 2.8-fold higher than in 4- and 12-d-old seedlings and roots, respectively (data not shown). Compared with Col-0, we found small changes in the expression of the RpoT genes in sca3-2/sca3-2 seedlings, rosettes, and roots. In sca3-2/sca3-2 individuals, RpoTm and RpoTmp showed higher transcript levels than in Col-0 in 4-d-old seedlings and 21-d-old plants, whereas a reduction was found for both genes in 12-d-old seedlings (Table I). SCA3 was down-regulated in the mutant in all developmental stages studied, especially in 4-d-old seedlings. Root transcript levels of the RpoT genes were similar in sca3-2/sca3-2 and Col-0 plants, the only exception being SCA3, for which a 2-fold increase was detected in the mutant.

![Figure 2](link). Positional cloning and structural analysis of the SCA3 gene. A, Map-based strategy followed to identify the SCA3 gene. After studying 882 chromosomes, a total of 33 recombinant events (in parentheses) were identified in a region of 11.7 cM on chromosome 2, flanked by the PLS8 and nga1126 markers. A candidate interval of 70 kb was finally delimited, flanked by the CER460016 and CER429871 markers and encompassing the T29E15 and F27D4 bacterial artificial chromosome clones, which included the A2g24060 to A2g24200 annotated genes. B, Structure of the SCA3 gene, with indication of the position and nature of the sca3 mutations. Exons and introns are indicated by black boxes and lines, respectively. Triangles indicate T-DNA insertions. Horizontal arrows indicate the oligonucleotides used to characterize the structure and expression of SCA3, which are not drawn to scale. C, Alignment of the 16th exon-intron junction region of the SCA3 gene in the wild-type Ler and the sca3-1 mutant. The sequences shown correspond to the splicing pattern found in the wild-type SCA3 allele (Ler) and the two detected in the sca3-1 mutant (here named sca3-1a and sca3-1b). Upper- and lowercase letters indicate exonic and intronic sequences, respectively. The eight-nucleotide segment absent from sca3-1b mRNA is underlined. An arrowhead indicates the single-nucleotide change found in genomic DNA. The cryptic splicing donor site within exon 16 used in sca3-1b is boxed. D, Alignment of the amino acid sequences corresponding to the C-terminal part of the protein products of the wild-type SCA3 and the mutant sca3-1 alleles of the SCA3 gene. An asterisk denotes a stop codon.
Effects of sca3 Mutations on Expression of Plastid Genes

Tobacco plastid genome genes have been classified into three classes, depending on which RNAP transcribes them: PEP only (class I), PEP and NEP (class II), or NEP only (class III; Hajdukiewicz et al., 1997). To our knowledge, such a study has yet to be made in Arabidopsis. We decided to ascertain whether the expression of plastid genes containing putative NEP-responsive sequences in their promoters was compromised in the sca3 mutants. qRT-PCR amplifications were performed on RNA extracted from 4- and 12-d-old seedlings and 21-d-old rosettes of Col-0 and the sca3-2 mutant (Table I) to quantify expression of the following plastid genes: clpP (assumed to be class II), rpoB, rpoC1, accD (assumed to be class III), and rps18 (not classified, given that its transcription initiation sites and promoters have not been mapped). These genes respectively encode the proteolytic subunit of the Clp ATP-dependent protease (Gray et al., 1990; Maurizi et al., 1990), the plastid b (Hu and Bogorad, 1990) and b# core subunits of PEP (Shinozaki et al., 1986), a subunit of the acetyl-CoA carboxylase involved in lipid biosynthesis (Sasaki et al., 1993), and a ribosomal protein (Shinozaki et al., 1986).

RpoTp seemed to be required for the transcription of all these plastid genes, as indicated by their significant down-regulation in 4-d-old seedlings of the sca3-2 mutant. In this mutant, only rpoB and rpoC1 were down-regulated in all the developmental stages studied, whereas the transcript levels of accD and clpP were reduced in 4-d-old seedlings, but increased in 12-d-old seedlings. In contrast, clpP transcript levels were reduced in sca3-2/sca3-2 rosettes and those of rps18 only in 4-d-old seedlings. No significant changes were found for accD in rosettes.

To ascertain whether the sca3 mutations affect plastid rRNA levels and consequently plastid ribosome abundance, total RNA was extracted from 21-d-old wild-type and sca3-1/sca3-1 and sca3-2/sca3-2 mutant plants, and their rRNAs were quantified after being visualized in a denaturating agarose gel stained by ethidium bromide (Supplemental Fig. 2). We found reduced signal intensities for the chloroplastic rRNAs of the sca3-2 mutant compared to Col-0, but no significant differences between sca3-1 and Ler (Supplemental Fig. 2). This is consistent with the molecular nature of the sca3-2 allele and its stronger mutant phenotype.

Phenotype of sca3 Mutants

Col-0 and Ler genetic backgrounds had no visible effect on the phenotypes of the sca3 mutants, as deduced from their comparison to the phenotypically mutant F2 progeny of their intercrosses. The sca3 mutants displayed pale-green cotyledons and vegetative leaves, particularly the sca3-2 and sca3-3 homozygotes, which showed yellowish cotyledons and leaves (Fig. 1, A–D). Consistent with the paleness of the sca3 mutants, we found a significant reduction in their chlorophyll content compared to Col-0, but no significant differences between sca3-1 and Ler (Supplemental Fig. 2). This is consistent with the molecular nature of the sca3-2 allele and its stronger mutant phenotype.
more reduced and leaf margins displayed deep serrations corresponding to the positions of hydathodes.

**Table 1. qRT-PCR analyses of the expression of RpoT and plastid genes**

Relative expression values were determined as $2^{-\Delta \Delta C_t}$ for each studied gene in the sca3-2 mutant after normalization with those of the OTC gene, and compared with those of wild-type Col-0, to which a value of 1 was given. Numbers in parentheses indicate the range of variation of $2^{-\Delta \Delta C_t}$ values of the studied gene obtained in two independent experiments using two different biological replicates and triplicate reactions. The range of variation of $2^{-\Delta \Delta C_t}$ values in Col-0 for the RpoTm, RpoTmp, SCA3, rpoB, accD, clpP, rps18, and rpoC1 genes, respectively, were as follows: 0.69 to 1.44, 0.66 to 1.50, 0.78 to 1.27, 0.74 to 1.33, 0.67 to 1.42, 0.63 to 1.58, 0.68 to 1.45, and 0.66 to 1.50 in 4-d-old seedlings; 0.72 to 1.38, 0.95 to 1.04, 0.65 to 1.81, 0.63 to 1.58, 0.97 to 1.02, 0.63 to 1.58, 0.87 to 1.14, and 0.96 to 1.01 in 12-d-old seedlings; 0.78 to 1.26, 0.46 to 2.15, 0.99 to 1.00, 0.59 to 1.67, 0.30 to 3.30, 0.82 to 1.20, 0.92 to 1.08, and 0.32 to 3.09 in rosettes. The range of variation for the RpoTm, RpoTmp, and SCA3 genes in Col-0 roots was 0.40 to 2.46, 0.33 to 3.00, and 0.40 to 2.48, respectively. N.D., Not determined.

**Ultrastructure of sca3/sca3 Leaves**

Scanning electron microscopy showed the surface of the mutant leaves to be wrinkled, extremely so in the case of the sca3-2 and sca3-3 homozygotes, whose lamina was completely crumpled (Fig. 4, A–D). Nevertheless, no obvious differences with the wild type were observed for the size and morphology of the sca3/sca3 adaxial and abaxial epidermal cells (Fig. 4, E–L).

We analyzed internal leaf anatomy by means of confocal microscopy of intact leaves (Fig. 5, A–H) and cross sections (Fig. 5, I–L) and found a reduced density of mesophyll cells in the sca3 mutants, particularly in sca3-2, whose intervascular areas and leaf margins were almost devoid of such cells. As a consequence, their leaf vascular network was distinguishable on a paler green background in intact leaves (Fig. 5, M–P). To ascertain whether this reduction was due to an increase in the frequency of cell death caused by sca3 mutations, we stained the mutants with trypan blue. No differences with the wild types were found (data not shown), which indicated that the number of dead cells was not increased by the sca3 mutations. In addition, mesophyll cells were irregularly shaped in the sca3-2 mutant (Fig. 5, K and L), making it impossible to distinguish between the palisade and spongy layers.

We examined leaf chloroplast ultrastructure in sca3-1/sca3-1, sca3-2/sca3-2, and wild-type individuals by transmission electron microscopy and found defects that paralleled the harshness of the morphological phenotype. Chloroplasts in the sca3-1 mutant displayed a reduced number of starch grains, but they were similar in size, morphology, and number to those of the wild type (Fig. 6, A and B). On the contrary, mesophyll cells in the sca3-2 mutant exhibited a large reduction in the number of chloroplasts, which, in
turn, lacked starch grains, were smaller, and showed a less-developed thylakoid organization (Fig. 6, A and C). Some chloroplasts of the sca3-2 mutant displayed enlarged thylakoid lamellas and transparent vacuoles (Fig. 6, A and D), a trait never observed in the wild type. Consistent with the small morphological effect of sca3 mutations on the vascular network, chloroplasts in the cells surrounding the midvein were found to be more similar to those of the wild type than those of the mesophyll cells (Fig. 6, A and C–E).

**Figure 4.** Scanning electron micrographs of sca3/sca3 leaves. Micrographs are shown of the adaxial surface (A–D) of whole leaves and details of their adaxial (E–H) and abaxial epidermis (I–L). All plants were homozygous for the mutations shown. Leaves were collected 28 d after sowing. Scale bars indicate 1 mm (A–D) and 10 μm (E–L).

**Figure 5.** Reduced cell density in the mesophyll of sca3/sca3 leaves. A to H, Confocal micrographs, showing fluorescing chlorophyll within mesophyll cells of whole third leaves (A–D), and details of the mesophyll (E–H). Transverse sections of leaves (I–L) and whole leaves (M–P). All plants were homozygous for the mutations shown. Leaves were collected 28 d after sowing. Scale bars indicate 1 mm (A–D), 50 μm (E–L), and 0.5 mm (M–P).
Photoautotrophic Growth in \textit{sca3} Mutants

It has been proposed that the chloroplastic NEP indirectly promotes expression of plastid genome photosynthetic genes through the activation of genes such as \textit{rpoB}, whose product is a PEP subunit (Hajdukiewicz et al., 1997). Together with the observed perturbation in plastid development, the pale-green pigmentation and reduction in growth of \textit{sca3} mutants suggest that SCA3 might be important for photoautotrophic growth. To test this hypothesis, we compared the growth of \textit{sca3} mutants in vitro in the presence or absence of 2% Suc in the culture medium. The absence of Suc did not perturb the growth of wild-type plants, but strongly affected that of mutants, especially \textit{sca3-2}, which displayed pale-green expanded cotyledons and a first pair of leaves of reduced size, a stage of arrested development that remained unchanged even 4 weeks after sowing (Supplemental Fig. 4). Consistent with this, the growth of \textit{sca3-2/sca3-2} and \textit{sca3-3/sca3-3} plants was seriously impaired when the seeds were sown on soil. Whereas all wild-type and mutant seeds reached 100% germination on agar medium including Suc, all of the wild-type seeds germinated and developed properly on soil, but only 19.0% of the \textit{sca3-2/sca3-2} and 36.7% of the \textit{sca3-3/sca3-3} seeds did so. These results indicate that SCA3 is required early in development to establish autotrophic growth. Similar results have been reported previously for the reticulate mutant \textit{cue1} (Li et al., 1995) and the pale mutant \textit{pac} (Reiter et al., 1994), whose leaf internal organization is perturbed.

Microarray Analysis of the \textit{sca3-2} Mutant

To examine the effect of \textit{sca3} mutations on the nuclear transcriptome of Arabidopsis, we performed a microarray analysis using RNA extracted from 3-week-old plants of the \textit{sca3-2} mutant and wild-type Col-0. Among the 26,173 genes represented on the DNA chip, 301 were found to be misregulated in the \textit{sca3-2} mutant. A total of 103 (34.2%) and 198 (65.8%) genes were at least 1.5-fold up-regulated or down-regulated, respectively (Supplemental Table III). Surprisingly, the most up-regulated gene found in the microarray was the MADS-box family member \textit{SEP3} (Mandel and Yanofsky, 1998; Pelaz et al., 2000). Consistent with the role of SCA3 in chloroplast biogenesis, 83 (27.6%) of the misregulated genes encoded proteins targeted to plastids. Given that the DNA chip contained the whole set of Arabidopsis nuclear genes, this proportion is significantly higher than that predicted for nuclear-encoded chloroplastic proteins, approximately 3,500 (13.7% of the genes in the array), according to the Arabidopsis Genome Initiative (Arabidopsis Genome Initiative, 2000). Among the genes encoding chloroplast-targeted proteins, the photosynthetic ones were the most widely represented (14; 16.8%), including genes encoding proteins of light-harvesting complexes (such as \textit{LHCB2}; Standfuss and Kühbrandt, 2004), as well as different subunits of the reaction center of PSI (such as \textit{PSAK}; Jensen et al., 2000) and PSII. Interestingly, 84.3% of the nuclear genes encoding chloroplast-targeted proteins were down-regulated in \textit{sca3-2}, which represents 35.0% of all the genes down-regulated, including all the photosynthetic genes and genes encoding FtsH proteases, the RNAP \sigma-subunit SigA (\textit{SIG1}; Isono et al., 1997), ATP-binding cassette transporters, and proteins involved in isoprenoid biosynthesis. The latter group included \textit{CLAI} (encoding the 1-deoxy-D-xylulose-5-P synthase; Mandel et al., 1996), \textit{ABSCISIC ACID1 (ABA1)}; zeaxanthin epoxidase; Audran et al., 2001; Xiong et al., 2001), \textit{NCED4} and \textit{CHLOROPLAST BIOGENESIS 4 [CLB4; hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase], and CLB6} (Gutiérrez-Nava et al., 2004). On the contrary,
among the up-regulated genes, only 12.6% corresponded to chloroplastic genes. We compared our results for the 20 genes that we found most down-regulated or up-regulated, as well as all the down-regulated photosynthetic genes, with those deposited at an Arabidopsis microarray database (http://www.genevestigator.ethz.ch). This comparison was made with the results obtained by previous authors in experiments performed using norflurazon, an inhibitor of carotenoid biosynthesis that blocks both chloroplast development (Susek et al., 1993) and expression of nuclear photosynthetic genes. All but one of the genes that we found down-regulated were also repressed in the presence of norflurazon. Other functional groups of genes identified in our microarray analysis included those involved in heat shock response (eight genes), defense (14), electron transport (16), and metabolism (34). The membrane transport (19), transcription regulation (31), modification (13), defense (14), electron transport (16), involved in heat shock response (eight genes), protein biosynthesis that blocks both chloroplast development (Susek et al., 1993) and expression of nuclear photosynthetic genes. All but one of the genes that we found down-regulated were also repressed in the presence of norflurazon. Other functional groups of genes identified in our microarray analysis included those involved in heat shock response (eight genes), defense (14), electron transport (16), membrane transport (19), transcription regulation (31), and metabolism (34). The RpoT genes were not found to be misregulated in the microarray, which is consistent with the small variation found between Col-0 and sca3-2/sca3-2 plants using qRT-PCR (Table I). Of the 301 genes identified in our analysis, 60 (20.0%) are of unknown function.

To validate our microarray results, we used qRT-PCR to analyze the expression of five genes in 3-week-old sca3-2/sca3-2 and Col-0 plants. SEP3 was 7.0- and 2.7-fold up-regulated in sca3-2 compared with Col-0, as detected by qRT-PCR and microarray analysis, respectively. In the sca3-2 mutant, the LCHB2, PSAK, SIG1, and ABA1 genes were respectively found 6.4-, 2.8-, 2.1-, and 1.6-fold down-regulated by qRT-PCR, whereas microarray analyses showed 3.7-, 2.1-, 1.7-, and 1.7-fold levels.

Double-Mutant Analysis

To gain insight into the role of the RpoT genes in plant development, the sca3-2 mutant was crossed to the rpoT;2 mutant, which carries a T-DNA insertion in the RpoTm gene (Baba et al., 2004). Both mutants were in a Col-0 genetic background. Because the rpoT;2 mutant was hard to distinguish from the wild type in our growth conditions, three phenotypic classes were found in the F₁ progeny, conforming to an expected ratio of 12:3:1 ($\chi^2 = 4.25; P = 0.05$). This included 165 phenotypically wild-type plants, 27 that displayed the phenotype of the sca3-2 parent and 14 that were considered double mutants, which was confirmed by genotyping their T-DNA insertions. Development of the sca3-2 rpoT;2 double mutants was arrested early after germination, when they displayed pale-green or bleached expanded cotyledons, and only a few of them produced a few leaves, which were extremely abnormal and tiny (Fig. 7).

DISCUSSION

The SCA3 Gene Encodes the RpoTp Protein

We describe here the positional cloning of the SCABRA3 gene of Arabidopsis and the characteriza-

Figure 7. Genetic interaction between the SCA3 and RpoTm genes. sca3-2 (A), rpoT;2 single mutants (B), and sca3-2 rpoT;2 double mutants (C and D). Images were taken 21 d after sowing. Scale bars indicate 1 mm.

RpoTp and RpoTm Are Partially Redundant in Green and Nongreen Tissues

The sca3 mutants displayed rounded and pale-green vegetative leaves with marked irregularities on the surface and the margins of the lamina, although leaf epidermal cell size and morphology were not perturbed. All the phenotypic traits studied were much more conspicuous in sca3-2 and sca3-3 than in sca3-1, which is consistent with their molecular nature. The sca3-1 allele carries a point mutation and encodes both a truncated protein lacking 109 amino acids and a wild-type protein. Each of the sca3-2 and sca3-3 insertional alleles encode a single protein, lacking 604 and 50 amino acids, respectively, and including divergent amino acids translated from the T-DNA. Because the protein product of sca3-2 lacks many more amino acids than that of sca3-3, the latter might represent an example of a mutation perturbing a redundant functional activity, such as that of RpoTm. This has been hypothesized for the recessive sleep1-1-2 (sly1-2) and sly1-10 alleles of the Arabidopsis gibberellin-signaling SLEEPY1 gene, which interfere with the function of the homologous SNEEZY gene (Strader et al., 2004).

Internal structure is severely perturbed in sca3/sca3 leaves as a consequence of a strong reduction in the
number of mesophyll cells and an increase in intercel-
lar airspaces, which presumably cause the surface
irregularities that characterize their external morphol-
ogy. Nevertheless, their leaf veins remained almost
unaltered, suggesting that sca3 mutations differential-
tially affect vascular and mesophyll cell development.
A transition from underdeveloped to normal meso-
phyll cells and from leaf margins to green sectors close
to the midvein has been reported for the virescent cue6
mutant of Arabidopsis (Lopez-Juez et al., 1998). Other
Arabidopsis mutants, such as cue1 (Li et al., 1995;
Streitfeld et al., 1999) and plastid protein import 2 (ppi2;
Asano et al., 2004), show reduced cell density in the
mesophyll and an associated alteration in leaf mor-
phology. The reduction in the number of mesophyll
cells in the sca3 mutants might be due to reduced cell
proliferation or, alternatively, to increased cell death.
The first of these two scenarios is more likely because
no difference was observed in the frequency of cell
death between sca3/sca3 and wild-type individuals.

Leaf epidermal cells seem unaffected in the sca3
mutants, which is remarkable given that plastids are
the primary site of amino acid and lipid biosynthesis.
Further research will be required to address the ques-
tion of whether RpoTp plays a more active role in the
mesophyll than in the epidermis, where RpoTmp
might be more important.

Roots contain photosynthetically inactive but differen-
 tiated plastids such as leucoplasts and amyloplasts
(Waters and Pyke, 2005). NEPs are assumed to tran-
scribe genes in nonphotosynthetic plastids to maintain
their metabolic activity, as well as being required in pro-
 plastids to keep them ready to differentiate into func-
tional chloroplasts (Magee and Kavanagh, 2002). In
addition, RpoTmp has been found not only in leaf chlo-
ropalast but also in nongreen root plastids (Hedtke
et al., 1999). Consistent with a function for RpoTp in
nongreen tissues, we detected SCA3 expression in roots
and found a significant reduction in root length and sec-
ondary roots in the strong sca3-2 mutant. The rpoT;2
mutant also exhibits short roots (Baba et al., 2004), which,
taken together with our results, suggests overlapping
functions for RpoTmp and RpoTp in root plastids.

sca3 mutations perturb other aspects of plant develop-
ment, causing a general reduction in size, stem and
silique length, and a generalized loss of pigmentation.
The small size of most organs of the sca3 mutants sug-
gests a role for SCA3 in promoting cell proliferation
not limited to leaf mesophyll cells. Reduced growth,
delayed greening, and wrinkled leaf lamina are also
characteristic traits of the rpoT;2 mutant (Baba et al.,
2004). Given that RpoTmp and RpoTp are homologous
and chloroplast targeted, our results suggest that they
are involved in similar developmental events and that
they are partially redundant. Consistent with this, we
found that the double mutant sca3-2 rpoT;2 is seedling
lethal.

The phenotype of sca3 mutants is enhanced by
growth at low temperature, as previously described
for mutations affecting chloroplast and mesophyll de-
velopment, such as var2 (Chen et al., 2000; Takechi
et al., 2000) and chilling-sensitive5 (chs5; Schneider et
al., 1995), which is allelic to clad (Araki et al., 2000).
Cold sensitivity of the sca3 mutants might be explained by
a reduction in the activity of SCA3 mutant proteins at
the restrictive temperature, which would require the
existence of some degree of remnant activity in the
protein product of the strong sca3-2 and sca3-3 alleles.
However, this activity would not be increased at 26°C
because the phenotype of the sca3-2/sca3-2 plants is
not suppressed as in sca3-1/sca3-1 individuals. In the
latter, the increased level of the wild-type splice form
found at 26°C might explain the suppression of the
mutant phenotype. Alternatively, the temperature sen-
sitivity of these mutants could be due to decreased
activity of a functionally redundant protein at the
restrictive condition. We find the latter hypothesis more
likely, given the extreme mutant phenotype, the mo-
 lecular nature of the sca3-2 and sca3-3 alleles, and the
fact that RpoTmp and RpoTp proteins share a sub-
stantial part of their structure and function. Given that
other mutants affected in chloroplast development
also show cold sensitivity, we cannot rule out the influ-
ence that enhanced photodynamic damage caused by
the restrictive temperature might produce on the phe-
notype of the sca3 mutants.

Perturbation of Chloroplast Development in sca3
Mutants Impairs Mesophyll Cell Differentiation

Chloroplasts were dramatically reduced in number
and not properly developed in leaves of sca3-2/sca3-2
plants, indicating that SCA3 activity is essential for chlo-
roplast development and suggesting a role for RpoTp
in transcribing the plastid genes required for the con-
version of proplastids into functional chloroplasts. Arabi-
dopsis variegated mutants such as im (Josse et al.,
2000; Aluru et al., 2001), var1, and var2 (for review, see
Sakamoto, 2003) also display abnormal, vacuolated
plastids in white leaf sectors. As a likely consequence
of the perturbation in plastid development, we found
that photoautotrophic growth is severely impaired in
the strong sca3-2 and sca3-3 mutants.

Several mutants with altered internal leaf anatomy
and chloroplast biogenesis have been described, sug-
gesting the existence of a putative plastid-to-nucleus
developmental signal that controls mesophyll cell pro-
liferation and differentiation. sca3 mutants provide fur-
ther support for the hypothesis that perturbation of
chloroplast biogenesis affects mesophyll cell differen-
tiation and hence leaf morphogenesis. Although the
existence of a plastid developmental signal controlling
leaf morphogenesis is yet to be demonstrated, a study
of the genomes uncoupled (gun) mutants of Arabidopsis
identified magnesium-protoporphyrin IX as one of the
plastid signals that regulates expression of nuclear
photosynthetic genes (Mochizuki et al., 2001; Surpin
et al., 2002; Strand et al., 2003; Strand, 2004). In our
microarray analysis of the sca3-2 mutant, we found sev-
eral photosynthetic genes significantly down-regulated,
including *LHCB2*, whose expression is known to be reduced in other mutants with abnormal leaf anatomy (Rodermel, 2001). Other down-regulated genes were *ABA1*, *CLAI*, *CLB4*, and *CLB6*, which are related to isoprenoid biosynthesis, a group of molecules with essential functions in photosynthesis, plant growth, and development (Peñuelas and Munne-Bosch, 2005). Another misregulated gene was *NCED4*, whose function is unknown and belongs to the 9-cis-epoxydocarotenoid dioxygenase family. Another member of this family, *NCED3* (Tan et al., 2003), participates in ABA biosynthesis, which also requires *ABA1* (Audran et al., 2001; Xiong et al., 2001). *ABA* is an abiotic stress response hormone (Zhu, 2002) that also acts as a positive regulator of plant growth (Cheng et al., 2002; González-Guzmán et al., 2002; Barrero et al., 2005) and seems to be involved in plastid differentiation (Rohde et al., 2000). On the other hand, the *CLAI*, *CLB4*, and *CLB6* genes are required for chloroplast development and function (Estévez et al., 2000; Gutiérrez-Nava et al., 2004). Therefore, our results are consistent with those obtained in other Arabidopsis pigment mutants with abnormal leaf morphogenesis and suggest the existence of two different plastid-to-nucleus signaling pathways, one controlling cell differentiation and leaf development and the other controlling expression of nuclear photosynthetic genes (Rodermel, 2001). We considered as an alternative hypothesis that the reduction in cell viability and vigor found in the *sca3* mutants might be due to a decrease in the expression of *ycf1* and *ycf2*, the two largest open reading frames encoded by the chloroplast genome of dicotyledonous plants, which are cell essential (Drescher et al., 2000), but our expression analyses ruled this out (data not shown).

Expression of *RpoT* Genes Is Not Strongly Affected by *sca3* Mutations

*SCA3*, *RpoTM*, and *RpoTmp* expression was detected in seedlings, roots, and rosettes of wild-type plants. We found small variations in *RpoTM* and *RpoTmp* expression between Col-0 and *sca3-2/sca3-2* plants. Thus, the level of *RpoTM* and *RpoTmp* expression was slightly reduced in *sca3-2* 12-d-old mutant seedlings, but both genes were up-regulated earlier (4-d-old seedlings) and later (21-d-old rosettes) compared with the wild type. Consistent with this, an increase in the levels of *RpoTM* and *SCA3* (*RpoTp*) transcripts in leaves of the Arabidopsis *rpoT*2 mutant has been reported (Baba et al., 2004) and an accumulation of *RpoTp* and *RpoTM* transcripts has been found in ribosome-deficient leaves of the *albostrians* mutant of *H. vulgare* (Emanuel et al., 2004). These results would indicate the existence of a compensatory effect acting as a regulatory mechanism that would maintain chloroplast function in the presence of mutations impairing their development. Nevertheless, the differences found in the expression of the *RpoT* genes in the *sca3-2* mutant do not seem to be sufficient to compensate for the lack of *SCA3* function, given the pleiotropic phenotype of the *sca3* mutants.

Expression of Plastid Genes Is Affected by *sca3* Mutations

It has been proposed that orthologs of the Arabidopsis *RpoTp* gene control the expression of a subset of genes of the plastid genome, those containing NEP-type promoters (Allison et al., 1996; Hajdukiewicz et al., 1997; Magee and Kavanagh, 2002), such as some housekeeping genes and PEP core subunits. According to this model (Hajdukiewicz et al., 1997), non-photosynthetic genes (such as *accD*, *clpP*, and others) and PEP components (such as the *rpoB* gene) are induced by NEP early in plastid differentiation. In later stages, light-dependent expression of sigma factors, such as SIG2, produces activation of PEP and triggers the differentiation of proplastids into functional chloroplasts (Hanaoka et al., 2005). At this stage, photosynthetic and housekeeping genes would be transcribed mainly by PEP.

Expression analysis of some plastid genes assumed to be representative of class II (transcribed by PEP and NEP) and III (transcribed by NEP) indicated that *SCA3* is required early and later in development for the expression of some plastid genes, such as *rpoB*, *rpoC1*, and *clpP* in both seedlings and rosettes. We found the lowest level of plastid gene expression in *sca3-2/sca3-2* 4-d-old seedlings, indicating that RpoTp plays an important role very early in chloroplast and plant development. Nevertheless, we cannot rule out that other factors, such as a reduction in RNA stability and/or synthesis, might contribute to the observed decrease in the levels of the plastid genes studied.

We found increased *accD* and *clpP* transcript levels in *sca3-2/sca3-2* 12-d-old seedlings. Consistent with this, an accumulation of NEP-dependent plastid transcripts has been reported in seedlings of Arabidopsis mutants with altered PEP components, such as *sig2* (Kanamaru et al., 2001; Nagashima et al., 2004) and *sig6* (Ishizaki et al., 2005), or defective NEP transcriptional machinery (*RpoT*2; Baba et al., 2004). Because the *RpoTM* and *RpoTmp* transcript levels are not increased in the *sca3-2/sca3-2* 12-d-old seedlings, the observed rise in *accD* and *clpP* transcript levels was probably not due to a compensatory up-regulation of the *RpoT* genes. Alternatively, an increase in the stability of the *accD* and *clpP* transcripts or PEP activity would compensate for the loss of RpoTp function in *sca3-2/sca3-3* seedlings, at least in the case of *clpP*, which is PEP responsive. This compensatory mechanism would be absent or less efficient later in development because the *clpP* mRNA levels decrease in *sca3-2* mutant rosettes.

The strong *sca3-2* mutation also causes a decrease in plastid rRNA levels and, probably, in the abundance of plastid ribosomes, which is consistent with the dramatic reduction in the number of chloroplasts found in this mutant. However, we cannot exclude the possibility that RpoTp might be required for the transcription...
of plastid rRNAs, given that mutants defective in chloroplast rRNA processing, maturation, or both, and displaying abnormalities in leaf development, have already been reported in Arabidopsis (Bellaoui et al., 2003; Bisanz et al., 2003; Kishine et al., 2004).

CONCLUSION

Mutations in SCA3 negatively affect the expression of both photosynthetic nuclear genes and plastid genes (the latter probably through the control of rpoB and rpoC1 by SCA3), resulting in abnormal chloroplast development and impaired photoautotrophic growth. In contrast to the role assigned to RpoTmp in early seedling development, characterization of sca3 mutants indicates that RpoTp is required both in early and late stages of vegetative development in Arabidopsis. Consistent with this, it has recently been found (Emanuel et al., 2005) that SCA3 (RpoTp) transcripts accumulated at similar levels early (cotyledons) and late (leaf tissue) during vegetative development and impaired photoautotrophic growth. Our findings highlight the complexity of the mechanisms that control plastid gene transcription.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cultures and crosses were performed as described in Ponce et al. (1998) and Berna et al. (1999), respectively. Seeds of the Arabidopsis (Arabidopsis thaliana L. Heynh.) wild-type accesses Ler and Col-0 were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The sca3-1 mutant was isolated in a Ler background after EMS mutagenesis (Berna et al., 1999). Seeds of several T-DNA insertion lines, including N567191 (sca3-3) and N593884 (sca3-2), were provided by the NASC and are described at the SGNWeb site (Alonso et al., 2003; http://signal.salk.edu). Seeds of the rpoT2.2 mutant were kindly provided by Julien Schmidt (Umea Plant Science Centre).

Growth Assays and Pigment Extraction

For temperature-sensitivity analyses, seeds were sown on agar plates as described above, incubated at 16°C, 20°C, or 26°C and observed 21 d after sowing. For root studies, seedlings were grown vertically on agar plates that were kept in the dark (wrapped in aluminum foil) for 2 weeks. For pigment extraction, 3-week-old plants were harvested and frozen in liquid nitrogen. Eighty milligrams (fresh weight) from each sample (four to eight individuals) were ground in chlorophyll and carotenoids were extracted adding 3.5 mL of 80% acetone to each sample. Pigments were quantified as described previously (Rabinov and Mancinelli, 1988). A Student’s t test was applied to the data obtained.

Morphological and Ultrastructural Analyses

Whole-rosette and single-leaf images were taken using a Leica MZ6 stereomicroscope equipped with a Nikon DXM1200 digital camera. Confocal imaging and trypan blue staining were performed as described in Pérez-Pérez et al. (2002) and Koch and Stusarenko (1990), respectively.

For light microscopy, plant material was fixed with formaldehyde-acetic acid/Triton (1.85% formaldehyde, 45% ethanol, 5% acetic acid, and 1% Triton X-100), as described in Serrano-Cartagena et al. (2000). Transverse sections of leaves (0.5-µm thick) were cut on a microtome (HM350S; Microm International), stained with 0.1% toluidine blue, and observed using a Leica DMRB microscope equipped with a Nikon DXM1200 digital camera under bright-field illumination.

For scanning electron microscopy, plant material was prepared as described in Serrano-Cartagena et al. (2000). Micrographs were taken in a JSM-840 FEOL scanning electron microscope. For transmission electron microscopy, mutant and wild-type plant material was harvested at exactly the same time of the day and fixed with 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2-7.4), washed with cacodylate-Suc buffer, and then postfixed in 1% (v/v) OsO4 (Wanson and Drochmans, 1968). The fixed specimens were dehydrated in an ethanol series (30%, 50%, 70%, and 100% ethanol; 30 min each) and embedded in LR White resin. Polymerized blocks were sectioned on a Reichert-Jung Ultratoc E microtome, stained with uranyl acetate and lead citrate, and visualized at 60 kV using a Zeiss EM10C transmission electron microscope.

Positional Cloning and Molecular Characterization of sca3 Mutations

To positionally clone the SCA3 gene, simple sequence-length polymorphisms, single-nucleotide polymorphisms, and cleaved-amplified polymorphic sequence markers were designed according to the polymorphisms between the Lc and Col-0 described at the Monsanto Arabidopsis Polymorphism Collection database (http://www.arabidopsis.org). For allelic sequencing, PCR products spanning the A2g24120 transcription unit were obtained using a template wild-type and mutant genomic DNA and the oligonucleotide primers shown in Supplemental Table I. Sequencing reactions were carried out with ABI PRISM BigDye Terminator cycle sequencing kits in 5-µL reaction volumes. Sequencing electrophoreses were performed on an ABI PRISM 3100 genetic analyzer.

RNA Extraction and Analyses

Unless otherwise stated, all RNA extractions from plant material (50-100 mg), RTs using random primers, and PCR amplifications of first-strand cDNA were performed as described in Quesada et al. (1999). For chloroplast rRNA analysis, 8 µg of total RNA from each sample were fractionated in formaldehyde-agarose gels. For detection of the different SCA3 transcripts in the sca3-1 mutant, SCA3 expression was tested by using a fluorescence-based semiautomated method (Ponce et al., 2000). In brief, total RNA was extracted from 3-week-old Ler and sca3-1/sca3-1 plants grown at either 20°C or 26°C, and RT-PCR amplification products were obtained using oligonucleotide primers flanking the sca3-1 mutation (F9, which had been labeled with 6-FAM phosphoramidite, and R10; Supplemental Table I) and electrophoresed in an ABI PRISM 3100 genetic analyzer (Supplemental Fig. 1).

qRT-PCR

Total RNA was extracted from 50 to 70 mg of 4- and 12-d-old seedlings and 3-week-old rosettes and roots (Col-0 and sca3-2) and DNase I treated using the Qiagen RNeasy plant mini kit, following the manufacturer’s instructions. RNA was ethanol precipitated and resuspended in 40 µL of RNase-free water. Five micrograms from each sample were reverse transcribed using random primers as described by Quesada et al. (1999) and 1 µL of the resulting cDNA solution was used for qRT-PCR amplifications, which were carried out in an ABI PRISM 7000 sequence detection system (Applied Biosystems) as described in Pérez- Pérez et al. (2004). Primer pairs (Supplemental Table I) were designed to yield amplification products of approximately 100 bp. One of the primers of each pair contained the sequences of the ends of two contiguous exons so that genomic DNA could not be amplified. Each 25-µL reaction mix contained 12.5 µL of the SYBR-Green PCR master kit (Applied Biosystems), 0.4 µM of primers, and 1 µL of cDNA solution. Relative quantitation of gene expression data was performed using the 2-ΔΔCt comparative Ct method (Livak and Schmittgen, 2001). Each reaction was performed in three replicates and levels of expression were normalized by using the Ct values obtained for the housekeeping OTC gene (Quesada et al., 1999).
Microarray Analysis

Arabidopsis wild-type Col-0 and mutant sca3-2/sca3-2 3-week-old plants grown in vitro from six different sowings (80–100 mg sample) were frozen in liquid N2 and ground by mortar and pestle. Total RNA was extracted as described in Quesada et al. (1999) and the six RNA samples obtained were pooled in pairs to generate three biological replicates. Ten micrograms of total RNA from each biological replicate were used for microarray hybridization and analysis. Superamine Telechem slides containing more than 26,000 spots corresponding to the Arabidopsis oligo set from Qiagen-Operon, obtained from David Galbraith (Arizona University; http://ag.arizona.edu/microarray), were hybridized by conventional methods with RNA probes labeled with either Cy3 or Cy5 Mono N-hydroxysuccinimide esters. Printed slides were rehydrated over a 65°C water bath for 10 s and dried on a 65°C heating block for 10 s. This hydration step was repeated three times. Oligonucleotides were fixed by 120 mJ of UV radiation. Slides were washed in 1% SDS for 5 min, in water for 5 min, and in absolute ethanol for 30 s. Finally, slides were dried by centrifugation at 141 g for 3 min.

Slides were then incubated at 37°C in 5× SSC, 0.1% SDS, twice in 0.5× SSC, then once in 0.1 M Na2CO3 (pH 9.0), labeled with either Cy3 or Cy5 Mono N-hydroxysuccinimide ester, and purified with Megaclear (Ambion), according to the manufacturer’s instructions. For each hybridization, 200 pmol of Cy3- and Cy5-labeled probes were mixed, dried under vacuum, and resuspended in 9 mL of RNase-free water. Labeled aRNA was fragmented by adding 1 mL of 10× fragmentation buffer (Ambion) and incubated at 70°C for 15 min. The reaction was stopped with 1 mL of Stop solution (Ambion). Integrity and average size of total RNA, aRNA, and fragmented aRNA was evaluated using Bioanalyzer 2100 (Agilent). Average size of aRNAs was about 1,000 nucleotides and of fragmented aRNAs 100–300 nucleotides. The probe was finally diluted to 100 ng/mL in hybridization buffer. Prehybridization was performed at 42°C for 30 to 45 min in 6× SSC, 0.5% SDS, and 1× bovine serum albumin, and slides were rinsed five times with water. Cy5 and Cy3 aRNA fragmented probes were mixed (200 pmol of each label) with 20 μg of PolyA (Sigma) and 20 μg of yeast (Saccharomyces cerevisiae) tRNA (Sigma) in a final volume of 90 μL of hybridization buffer (50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt’s). The probe was denatured at 95°C for 5 min and poured into the slide using a LifterSlip (Erie Scientific). Slides were then incubated at 37°C for 16 h in hybridization chambers (Array-It) and then sequentially washed in the following solutions: twice in 0.5× SSC, 0.1% SDS, twice in 0.5× SSC, and finally in 0.05× SSC for 5 min each. Slides were finally dried by centrifugation at 56°C for 1 h before being scanned.

Images from the Cy3 and Cy5 channels were equilibrated and captured with a GenePix 4000B (Axon) and spots quantified using GenePix software (Axon). The data from each scanned slide were first escalated and normalized using the Lowess method and then log transformed to correct the artifacts inherent in labeling, hybridization, scanning, and quantification, and analyzed by using the SOLAR package (BioAlma; http://www.bioalma.com). Two statistical approaches were used to identify differentially regulated genes: a t test (Smyth et al., 2002) and a z score (Quackenbush, 2002). Only genes with a signal > 50, P value < 0.05, z-score > 1.96 or < -1.96, and fold change > 1.5 or < -1.5 were considered differentially expressed.

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LITERATURE CITED


The Arabidopsis SCABRA3 Gene

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