The *SCABRA3* Nuclear Gene Encodes the Plastid RpoTp RNA Polymerase, Which Is Required for Chloroplast Biogenesis and Mesophyll Cell Proliferation in Arabidopsis[1][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 *RpoTm* 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* (*dal1*; Babiychuk et al., 1997; despite the sequence similarity between DAL1 and DAG proteins, the corresponding genes are not orthologs [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; Streatfield 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) mutant 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* (Emanuel 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 *rpoT;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 morphogenesis.

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* (L*er*) genetic background to Columbia-0 (Col-0). Linkage analysis of the F_2-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 F_1 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 L*er* 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*, L*er*, 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

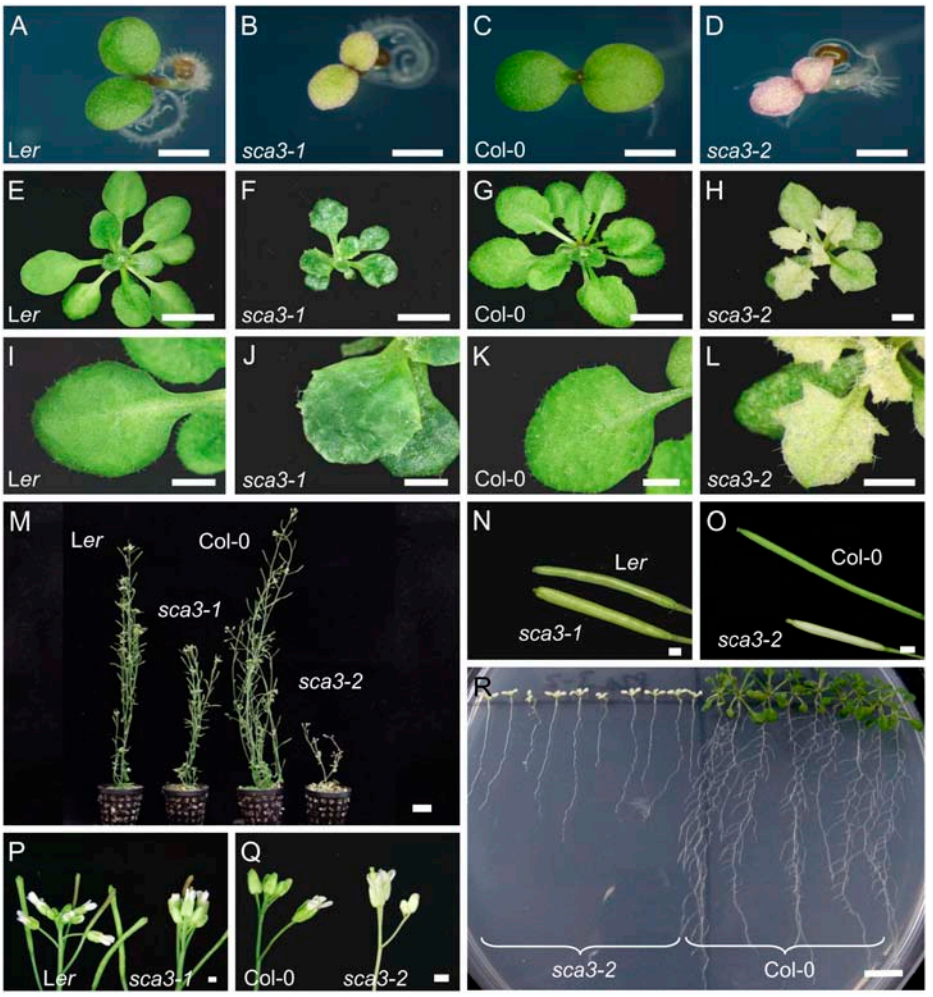


Figure 1. Some morphological traits of the phenotype of *sca3* mutants. Images correspond to cotyledons of 7-d-old seedlings (A–D), 21-d-old rosettes (E–H), and third node vegetative leaves (I–L) of the *sca3-1* and *sca3-2* mutants and their corresponding wild types (L*er* and Col-0, respectively). Six-week-old plants (M) grown on soil and details of their siliques (N and O) and inflorescences (P and Q). R, Fifteen-day-old plants vertically grown on agar medium. All plants were homozygous for the mutations shown. Scale bars indicate 1 mm (A–D, H–L, and N–Q), 5 mm (E–G), 10 mm (R), and 25 mm (M).

were obtained from *sca3-1*/*sca3-1* than from L*er* 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

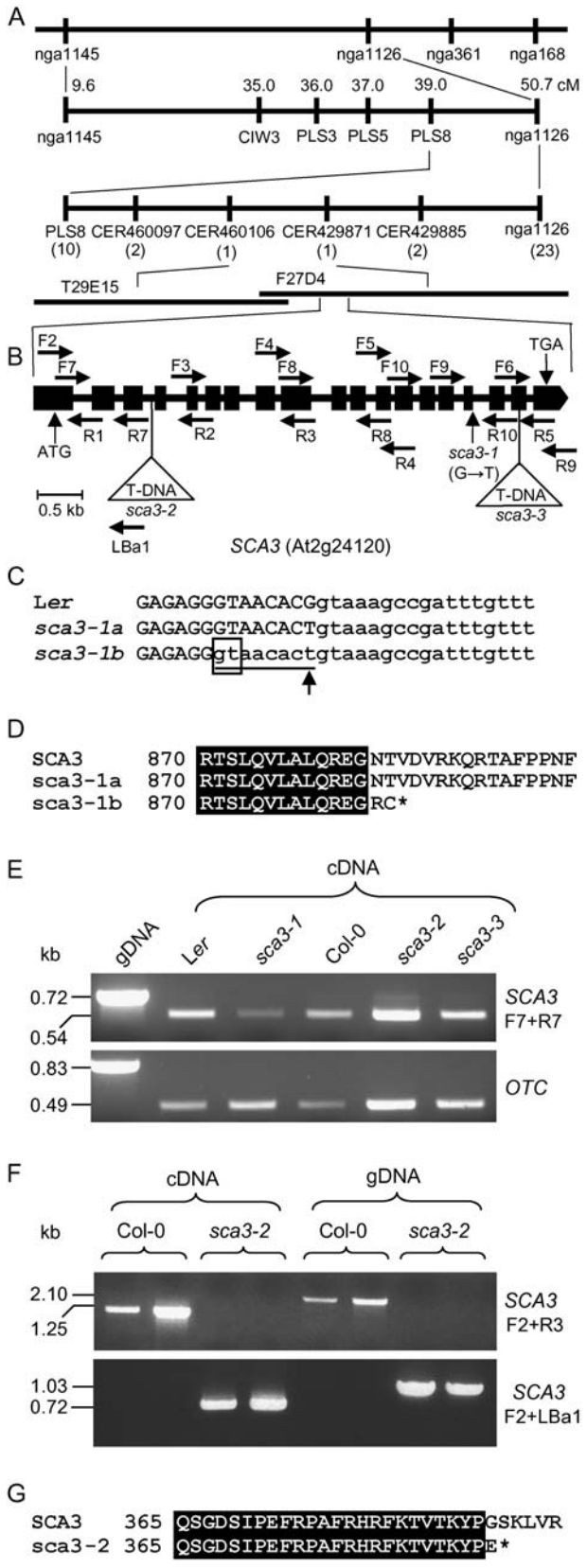


Figure 2. Positional cloning and structural analysis of the *SCA3* gene. A, Map-based strategy followed to identify the *SCA3* gene. After studying 882 chromosomes, a total of 33 recombinant events (in parentheses) were identified in a region of 11.7 cM on chromosome 2, flanked by the PLS8 and nga1126 markers. A candidate interval of 70 kb was finally delimited, flanked by the CER460106 and CER429871 markers and encompassing the T29E15 and F27D4 bacterial artificial chromosome clones, which included the At2g24060 to At2g24200 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 sixteenth exon-intron junction region of the *SCA3* gene in the wild-type L*er* and the *sca3-1* mutant. The sequences shown correspond to the single splicing pattern found in the wild-type *SCA3* allele (L*er*) 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 *sca3-1* 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. E, Expression analysis of the *SCA3* gene by RT-PCR in *sca3*/*sca3* and wild-type individuals. The bands shown were obtained after PCR amplifications were performed using as a template genomic DNA (gDNA) or RNA extracted from 3-week-old plants and reverse transcribed, and the primers shown. The *OTC* gene was used as an internal control. F, Expression analysis of the *SCA3* gene by RT-PCR in *sca3-2*/*sca3-2* and Col-0 individuals. The bands shown were obtained after PCR amplifications performed using as a template gDNA or RNA extracted from 3-week-old plants and reverse transcribed, and the primers shown. G, Alignment of the amino acid sequences corresponding to the protein products of the wild-type *SCA3* and the mutant *sca3-2* alleles of the *SCA3* gene. An asterisk denotes a stop codon.

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 downregulated 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.

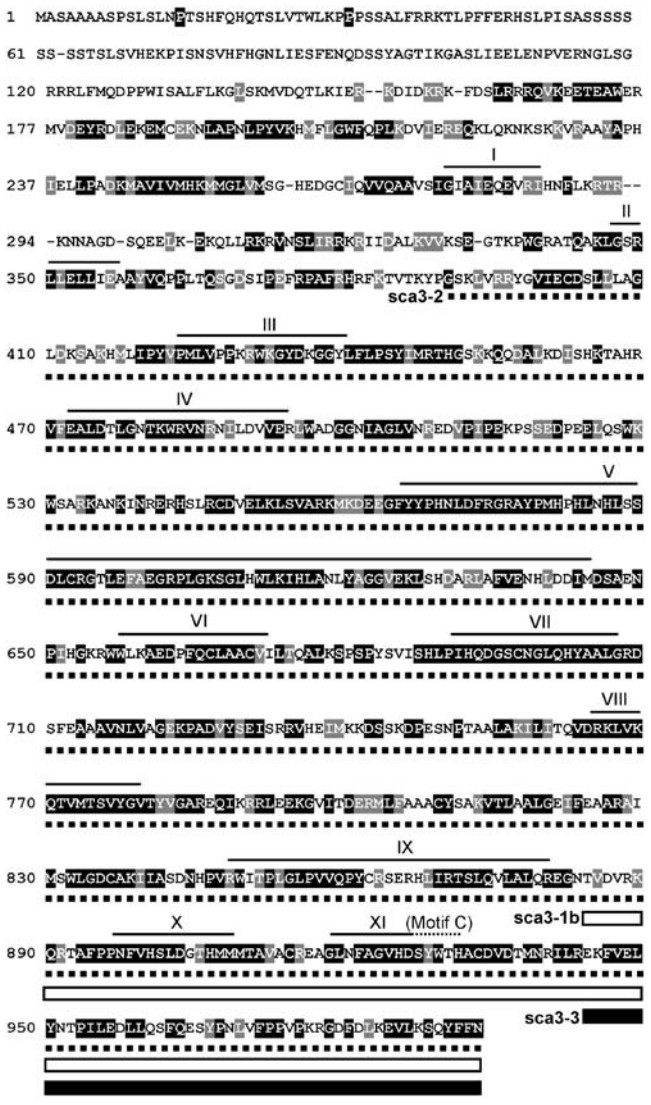


Figure 3. Predicted amino acid sequence of the Arabidopsis SCA3 protein (RpoTp; Y08463). Residues shaded in black are identical across the sequences of homologous gene products from *H. vulgare* (CAD45446), *T. aestivum* (AF091839), *Z. mays* (AF127022), *O. sativa* (AB120430), *N. sylvestris* (AJ302020), and tobacco (AJ416570). Residues with similar chemical properties conserved across all seven sequences are shaded gray. The alignment was obtained using ClustalX, version 1.5b. Continuous and dotted thin lines indicate the domains (I–XI) and the C-motif that are conserved among T7 phage-type NEP plastid RNAPs, respectively (McAllister and Raskin, 1993; Chang et al., 1999). The amino acid sequences deleted in the mutant protein products of *sca3-1b* (see Fig. 2), *sca3-2*, and *sca3-3* are indicated by white boxes, thick discontinuous lines, and black boxes, respectively.

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 β (Hu and Bogorad, 1990) and β' 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 L*er* (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 L*er* genetic backgrounds had no visible effect on the phenotypes of the *sca3* mutants, as deduced from their comparison to the phenotypically mutant F_2 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 the wild types, the differences being more pronounced in the *sca3-2* mutant, which also showed lower amounts of carotenoids (Supplemental Table II). The vegetative leaves of the *sca3-1* mutant were rounded, slightly reduced in size and petiole length, and presented lateral teeth (Fig. 1, I–L). In the *sca3-2* and *sca3-3* mutants, leaf size was much

Table I. *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, 0.55 to 1.81, 0.63 to 1.58, 0.97 to 1.02, 0.63 to 1.58, 0.87 to 1.14, and 0.98 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.

Genes	Normalized Transcript Levels in the *sca3-2* Mutant Relative to Col-0			
	4-d-Old Seedlings	12-d-Old Seedlings	Rosettes	Roots
RpoTm	1.97 (1.32–2.95)	0.67 (0.49–0.92)	1.50 (1.40–1.60)	1.10 (0.34–3.60)
RpoTmp	1.45 (0.99–2.12)	0.74 (0.71–0.77)	1.38 (0.68–2.80)	1.03 (0.32–3.31)
SCA3	0.32 (0.31–0.33)	0.95 (0.64–1.40)	0.72 (0.66–0.80)	2.07 (1.13–3.79)
rpoB	0.19 (0.05–0.74)	0.54 (0.31–0.95)	0.72 (0.26–1.96)	N.D.
accD	0.33 (0.23–0.48)	1.42 (1.02–1.97)	1.02 (0.36–2.87)	N.D.
clpP	0.34 (0.15–0.77)	2.12 (1.56–2.87)	0.44 (0.36–0.54)	N.D.
rps18	0.57 (0.45–0.77)	1.09 (0.97–1.23)	0.87 (0.79–0.96)	N.D.
rpoC1	0.33 (0.22–0.50)	0.78 (0.64–0.96)	0.65 (0.27–1.55)	N.D.

more reduced and leaf margins displayed deep serrations corresponding to the positions of hydathodes.

sca3 mutants displayed pale-green organs and a general reduction of growth, as seen from their short stems and petioles and small cauline leaves and siliques (Fig. 1, M–Q). Apart from the paleness of the sepals, we found no other obvious alteration in their floral organs. All these phenotypic traits were more extreme in the *sca3-2* and *sca3-3* mutants. For instance, root length was 7.5 ± 1.7 cm for L*er*, 6.1 ± 1.6 for *sca3-1/sca3-1*, 6.9 ± 2.0 for Col-0, and 3.6 ± 0.7 for *sca3-2/sca3-2*. In addition, *sca3-2/sca3-2* plants exhibited almost no lateral roots (Fig. 1R).

We found no differences in the photomorphogenic response of *sca3* mutants and their wild types grown for 2 weeks in the dark or after their return to normal light conditions (data not shown). This indicates that *SCA3* is not required to complete the etiolated developmental program, nor, therefore, for the function that etioplasts perform in the dark, at least at the stage analyzed.

The phenotype of the three *sca3* mutants studied here was cold sensitive. When grown at 16°C, they showed leaf chlorosis and were much smaller than the wild type (Supplemental Fig. 3). Growth at 26°C was similarly increased in wild-type and mutant plants compared to that observed at 20°C. In addition, the mutant phenotype of the *sca3-1/sca3-1* plants was partially suppressed at 26°C, at which temperature the irregularities of the leaf margin almost completely disappeared. This was consistent with the ratio of *SCA3* wild-type/mutant splice forms in *sca3-1/sca3-1* plants, which was found higher at 26°C (3.5 ± 1.8) than at 20°C (1.7 ± 0.4). On the contrary, apart from the increased growth, the phenotypic traits of the *sca3-2/sca3-2* plants grown at 26°C were not significantly different from that observed at 20°C (Supplemental Fig. 3).

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 interveinal 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

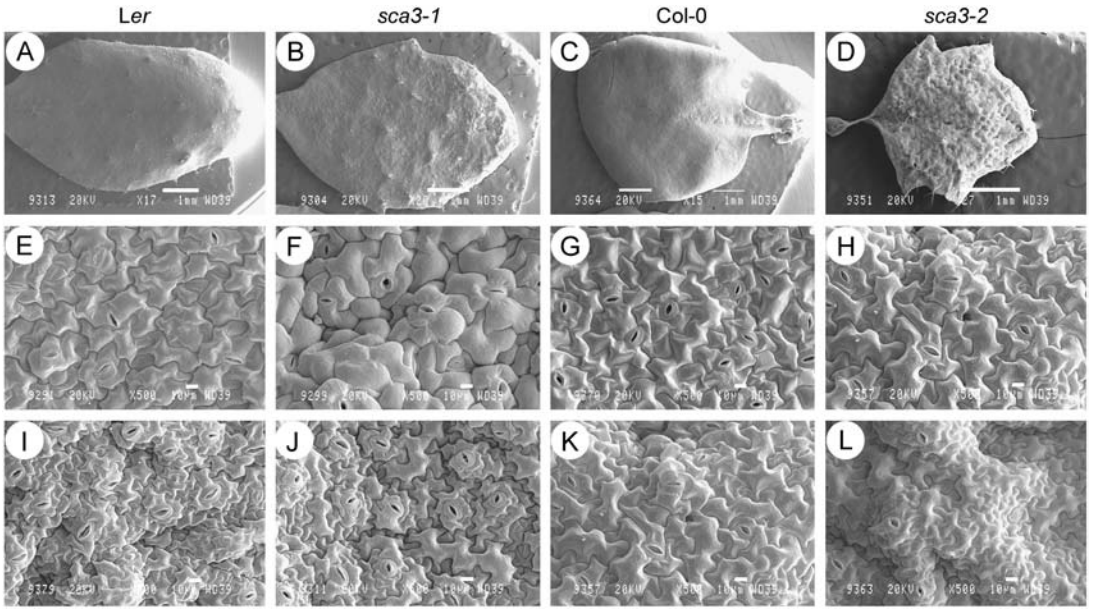
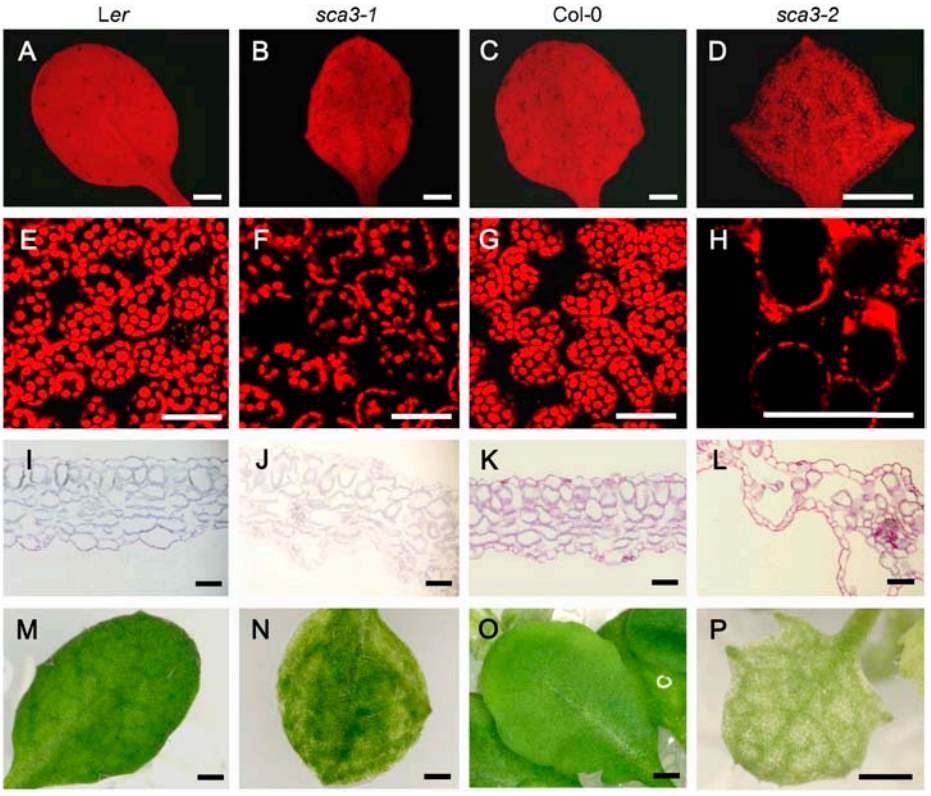


Figure 4. Scanning electron micrographs of *sca3*/*sca3* leaves. Micrographs are shown of the adaxial surface (A–D) of whole leaves and details of their adaxial (E–H) and abaxial epidermises (I–L). All plants were homozygous for the mutations shown. Leaves were collected 28 d after sowing. Scale bars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

turn, lacked starch grains, were smaller, and showed a less-developed thylakoid organization (Fig. 6, A and C). Some chloroplasts of the s*ca3*-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 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).



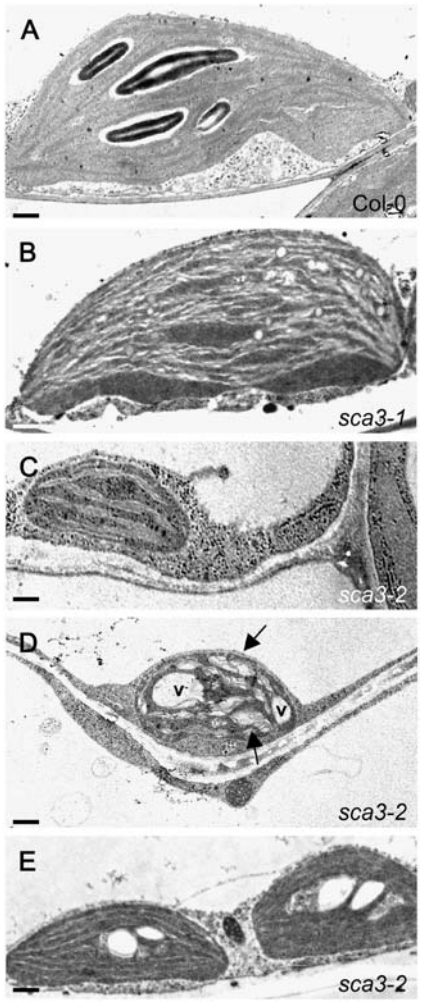


Figure 6. Transmission electron micrographs of *sca3*/*sca3* chloroplasts. A to C, Representative chloroplasts of Col-0 and the *sca* mutants. Chloroplasts of L*er* and Col-0 were indistinguishable and no differences were found between those of their mesophyll cells and those of cells neighboring the midvein. D, One of the chloroplasts occasionally seen in *sca3-2*/*sca3-2* mesophyll cells, showing enlarged thylakoids (arrows) and vacuoles (v). E, Chloroplasts of a cell adjacent to the midvein of the *sca3-2*/*sca3-2* mutant. All plants were homozygous for the mutations shown. All leaves were collected from the third node 21 d after sowing. Scale bars indicate 1.5 μm (A and B), 0.6 μm (C and D), and 1.2 μm (E).

Photoautotrophic Growth in *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 *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 *sca3* mutants suggest that *SCA3* might be important for photoautotrophic growth. To test this hypothesis, we compared the growth of *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 *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 *sca3-2*/*sca3-2* and *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 *sca3-2*/*sca3-2* and 36.7% of the *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 *cue1* (Li et al., 1995) and the pale mutant *pac* (Reiter et al., 1994), whose leaf internal organization is perturbed.

Microarray Analysis of the *sca3-2* Mutant

To examine the effect of *sca3* mutations on the nuclear transcriptome of Arabidopsis, we performed a microarray analysis using RNA extracted from 3-week-old plants of the *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 *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 *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 *LHCB2*; Standfuss and Kühlbrandt, 2004), as well as different subunits of the reaction center of PSI (such as *PSAK*; Jensen et al., 2000) and PSII. Interestingly, 84.3% of the nuclear genes encoding chloroplast-targeted proteins were down-regulated in *sca3-2*, which represents 35.0% of all the genes down-regulated, including all the photosynthetic genes and genes encoding FtsH proteases, the RNAP σ-subunit SigA (*SIG1*; Isono et al., 1997), ATP-binding cassette transporters, and proteins involved in isoprenoid biosynthesis. The latter group included *CLA1* (encoding the 1-deoxy-D-xylulose-5-P synthase; Mandel et al., 1996), *ABSCISIC ACID1* (*ABA1*; zeaxanthin epoxidase; Audran et al., 2001; Xiong et al., 2001), *NCED4* and *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), protein modification (13), 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 *RpoTmp* 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 F_2 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 characterization of three recessive *sca3* mutant alleles at the phenotypic, genetic, and molecular levels. We found that the protein product of *SCA3* is RpoTp, a nuclear-encoded, plastid-targeted RNAP. *SCA3* has two paralogs in the nuclear genome of Arabidopsis: *RpoTm* and *RpoTmp*, whose protein products are respectively targeted to mitochondria and to both plastids and mitochondria (Hedtke et al., 1997, 1999, 2000). A functional analysis has recently been reported for one of these genes, *RpoTmp* (Baba et al., 2004). Although homologs of the Arabidopsis *SCA3* gene have been characterized in several plant species, to our knowledge, the role of RpoTp in plant development has not been studied at a functional level using a mutational approach.

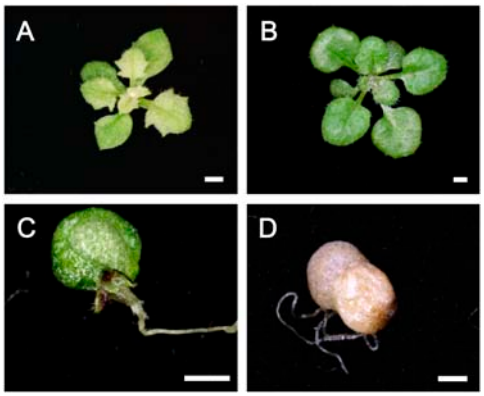


Figure 7. Genetic interaction between the *SCA3* and *RpoTmp* genes. *sca3-2* (A), *rpoT;2* single mutants (B), and *sca3-2 rpoT;2* double mutants (C and D). Images were taken 21 d after sowing. Scale bars indicate 1 mm.

RpoTp and RpoTmp 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 *RpoTmp*. This has been hypothesized for the recessive *sleepy1-2* (*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 intercellular airspaces, which presumably cause the surface irregularities that characterize their external morphology. Nevertheless, their leaf veins remained almost unaltered, suggesting that *sca3* mutations differentially affect vascular and mesophyll cell development. A transition from underdeveloped to normal mesophyll cells and from leaf margins to green sectors close to the midvein has been reported for the virescent *cue6* mutant of Arabidopsis (López-Juez et al., 1998). Other Arabidopsis mutants, such as *cue1* (Li et al., 1995; Streatfield et al., 1999) and *plastid protein import2* (*ppi2*; Asano et al., 2004), show reduced cell density in the mesophyll and an associated alteration in leaf morphology. 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 question 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 differentiated plastids such as leucoplasts and amyloplasts (Waters and Pyke, 2005). NEPs are assumed to transcribe genes in nonphotosynthetic plastids to maintain their metabolic activity, as well as being required in proplastids to keep them ready to differentiate into functional chloroplasts (Magee and Kavanagh, 2002). In addition, RpoTp has been found not only in leaf chloroplasts 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 secondary 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 development, 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 suggests 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 development, such as *var2* (Chen et al., 2000; Takechi et al., 2000) and *chilling-sensitive5* (*chs5*; Schneider et al., 1995), which is allelic to *cla1* (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 sensitivity 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 molecular nature of the *sca3-2* and *sca3-3* alleles, and the fact that RpoTmp and RpoTp proteins share a substantial part of their structure and function. Given that other mutants affected in chloroplast development also show cold sensitivity, we cannot rule out the influence that enhanced photodynamic damage caused by the restrictive temperature might produce on the phenotype 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 chloroplast development and suggesting a role for RpoTp in transcribing the plastid genes required for the conversion of proplastids into functional chloroplasts. Arabidopsis 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, suggesting the existence of a putative plastid-to-nucleus developmental signal that controls mesophyll cell proliferation and differentiation. *sca3* mutants provide further support for the hypothesis that perturbation of chloroplast biogenesis affects mesophyll cell differentiation 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 several 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*, *CLA1*, *CLB4*, and *CLB6*, which are related to isoprenoid biosynthesis, a group of molecules with essential functions in photosynthesis, plant growth, and development (Peñuelas and Munné-Bosch, 2005). Another misregulated gene was *NCED4*, whose function is unknown and belongs to the 9-cis-epoxycarotenoid 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 *CLA1*, *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), nonphotosynthetic 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*/*sca3-2* 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 *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, whereas *RpoTm* and *RpoTmp* were predominantly expressed early in Arabidopsis development. 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 Berná et al. (1999), respectively. Seeds of the Arabidopsis (*Arabidopsis thaliana* L. Heynh.) wild-type accessions L*er* and Col-0 were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The *sca3-1* mutant was isolated in a L*er* background after EMS mutagenesis (Berná 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 SIGnAL Web site (Alonso et al., 2003; http://signal.salk.edu). Seeds of the *rpoT;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 (15 seedlings/plate), photographed 15 d after sowing, and a Student's *t* test was applied to the data obtained. To study autotrophic growth, seeds were sown on petri dishes and plants grown in vitro in the presence or absence of 2% Suc in the culture medium, and the percentages of survival and arrested development were scored 21 d after sowing. To study the photomorphogenic response, seeds were sown 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 and chlorophyll and carotenoids were extracted adding 3.5 mL of 80% acetone to each sample. Pigments were quantified as already described (Rabino and Mancinelli, 1986). 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 Slusarenko (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 JEOL 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) OsO_4 (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 Ultracut 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 L*er* and Col-0 described at the Monsanto Arabidopsis Polymorphism Collection database (http://www.arabidopsis.org). For allele sequencing, PCR products spanning the At2g24120 transcription unit were obtained using as 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 L*er* and *sca3-1*/*sca3-1* plants grown at either 20°C or 26°C, and RT-PCR amplification products were obtained using oligonucleotides flanking the *sca3-1* mutation (F9, which had been labeled with 6-FAM phosporamidite, 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 quantification of gene expression data was performed using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen, 2001). Each reaction was performed in three replicates and levels of expression were normalized by using the C_T 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 N_2 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.

Total RNA (1 μg of each sample) was amplified and aminoallyl labeled using a MessageAmp II amplified RNA (aRNA) kit (Ambion) and 5-(3-aminoallyl)-2′deoxyuridine-5′-triphosphate (Ambion), according to the manufacturer's instructions, which yielded 40 to 50 μg of aRNA. For each sample, 7.5 μg of aminoallyl-labeled aRNA were resuspended in 0.1 M Na_2CO_3 (pH 9.0), labeled with either Cy3 or Cy5 Mono *N*-hydroxysuccinimide ester, and purified with Megaclear (Ambion), following the manufacturer's instructions. For each hybridization, 200 pmol of Cy3- and Cy5-labeled probes were mixed, dried in a speed vac, and resuspended in 9 μL of RNase-free water. Labeled aRNA was fragmented by adding 1 μL of 10 × fragmentation buffer (Ambion) and incubated at 70°C for 15 min. The reaction was stopped with 1 μL 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 nucleotides. The probe was finally diluted to 100 μL 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 (ArrayIt) 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 563*g* for 1 min 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 gene 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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