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Snowy cotyledon 2: the identification of a zinc finger domain protein essential for chloroplast development in cotyledons but not in true leaves

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Abstract In cotyledons of etiolated seedlings light-dependent transformation of etioplasts to chloroplasts marks the transition from heterotrophic to autotrophic growth. Genetic factors required for this developmental step were identified by isolating mutants of *Arabidopsis thaliana* that were impaired in chloroplast development in cotyledons but not in true leaves. Several mutants with chlorophyll-deficient cotyledons were isolated and dubbed *snowy cotyledon* (*sco*). Here we describe the identification and detailed characterization of the *snowy cotyledon 2* mutant. The mutated *SCO2* gene was identified using a map-based cloning strategy. *SCO2* was shown to encode a novel protein which contains a single DnaJ-like zinc finger domain. The SCO2 protein fused to GFP was shown to be present in chloroplasts. Inactivation of SCO2 has almost no detectable impact on the levels of transcripts encoding plastid-specific proteins but leads to a significant reduction of plastid protein levels. Even though transcripts of *SCO2* have been found ubiquitously in green tissues as well as in roots phenotypic changes due to *SCO2* inactivation are confined to cotyledons. The cotyledons in embryos of *sco2* are unaffected in their chloroplast biogenesis. Upon precocious germination seedlings of *sco2* and wild type are indistinguishable. The SCO2 mutation affects chloroplast biogenesis only at the end of dormancy during seed germination. The transition from heterotrophic to autotrophic growth is dramatically impaired in *sco2* when seedlings were kept in the dark for more than 5 days prior to light exposure.

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Introduction

Autotrophic growth of plants depends on their capacity to photosynthesize. During seedling development cotyledons initially serve as a storage organ that in the absence of light provides the young seedling with nutrients. Once the seedling emerges from the soil and is exposed to light its etiolasts are rapidly transformed to chloroplasts. This transition marks a major metabolic change from heterotrophic to autotrophic growth as revealed by both genetic and biochemical experiments. The light-dependent chloroplast development in cotyledons has been shown to be different in several ways from chloroplast development in true leaves. For instance, the import of the precursor of the NADPH-protochlorophyllide oxidoreductase PORA into plastids of cotyledons depends on the presence of protochlorophyllide, the immediate precursor of chlorophyllide, whereas in true leaves PORA import into chloroplasts occurs also in the absence of the tetrapyrrole intermediate (Kim and Apel 2004). Furthermore, several cotyledon-specific chloroplast biogenesis mutants have been described. Mutation of the white cotyledon gene (*WCO*) that affects the maturation of the 16SrRNA (Yamamoto et al. 2000) and impairment of the transcription factor sigma 2 gene (Privat et al. 2003; Shirano et al. 2000) or the plastid-specific elongation factor G gene all affect selectively chloroplast development in cotyledons (Albrecht et al. 2006). These mutations are different from those impeding chloroplast development not only in cotyledons but also in true leaves and that give rise to various *albino*, *xantha*, and *yellow green* mutants (Runge et al. 1995; Sundberg et al.

1997). The identification of these latter mutated genes led to the identification of components of the protein import machinery of chloroplasts, enzymes of chlorophyll biosynthesis and factors needed for the assembly of photosynthetic membranes (Asano et al. 2004; Chou et al. 2003; Gutensohn et al. 2004; Gutierrez-Nava Mde et al. 2004).

A mutant screen has been initiated that was aimed at identifying genes required for proper chloroplast development specifically in cotyledons. One of the mutants, *snowy cotyledon2* (*sco2*), has been characterized in the present work in greater details.

Results

Isolation and characterization of the *sco2* mutant

During germination one of the major characteristics of an autotrophic epigeous plant is the light-dependent formation of functional chloroplasts in cotyledons. In order to identify factors necessary for the regulation of chloroplast development in embryonic leaves, a mutant screen has been designed. Mutants affected in normal chloroplast development in cotyledons but not in true leaves were identified among M2 seedlings of 75,000 of ethylmethylsulfonate (EMS) mutagenized plants of *Arabidopsis thaliana* (ecotype Landsberg erecta). Mutants exhibiting pale cotyledons but normal green true leaves were selected. One of these mutants, *snowy cotyledon 1* (*sco1*) has recently been described (Albrecht et al. 2006). The mutation was identified in a gene encoding the plastid elongation factor G. In the present work another *snowy cotyledon* mutant, *sco2*, has been analyzed. In contrast to the pale cotyledons true leaves of *sco2* have a normal green appearance. Growth of the *sco2* mutant on sucrose-containing media does not affect the pale cotyledon phenotype (Fig. 1). Backcrossing of a homozygous *sco2* mutant with wild type revealed the recessive character of the *sco2* mutation with a Mendelian 1 : 3 segregation. A second mutant has been identified in

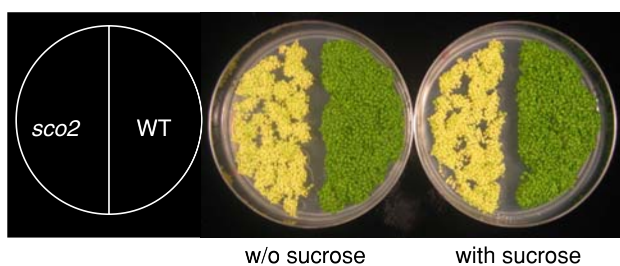

Fig. 1 The effect of sucrose on the phenotype of the *sco2* mutant. The pale cotyledons of *sco2* cannot be rescued by growth on MS media supplemented with 0.5 % sucrose. WT, wild type.

an independent screen in our laboratory that turned out to be allelic to *sco2*. Seedlings of the F1 generation of a cross of both homozygous mutant lines exhibited pale cotyledons.

The impairment of chloroplast formation in *sco2* has been described by measuring the chlorophyll content at different stages of seedling development and comparing it to that of wild type seedlings. Cotyledons of *sco2* accumulated only about 15 % of the chlorophyll of wild type in 3 day-old and 50 % of the chlorophyll content in 14 day-old seedlings (Fig. 2a). In contrast, true leaves of 14 day-old *sco2* seedlings accumulate the same amount of chlorophyll as wild type. Thus, in the *sco2* mutant accumulation of chlorophyll appears to be affected only in cotyledons but not in true leaves.

The impact of *sco2* on the assembly of the photosynthetic membrane was assayed by low temperature fluorescence measurements of frozen samples. 3 day-old and 7 day-old seedlings and cut cotyledons of 14 day-old seedlings were frozen in liquid nitrogen. The fluorescence emission spectra were measured using an excitation wavelength of 440 nm. An example of the resulting spectra for 3 day-old seedlings is shown in Fig. 2b. The fluorescence emission peak at 730 nm that reflects PS I is reduced in the *sco2* mutant relative to the fluorescence emission by PS II at 680 nm. Changes in the ratio of PS I and PS II during seedling development were calculated based on three independent measurements. In WT the ratio of PS I to PS II fluorescence increases during seedling development from about 3.5 in 3 day-old seedlings to up to 4.5 in cotyledons of 14 day-old seedlings. In contrast, in the *sco2* mutant the ratio of PS I to PS II fluorescence in 3 day-old seedlings is about 1 and increases to 3.4 in cotyledons of 14 day-old seedlings (Fig. 2c).

The expression of plastid genes in *sco2*

The impairment of chloroplast development in *sco2* is likely to be reflected also in the accumulation of plastid proteins and the expression of plastid genes. Total mRNA was extracted from 3 day-old seedlings and from cotyledons and true leaves of 14 day-old seedlings. Equal amounts of RNA were reverse-transcribed and transcript levels of different plastid genes were determined by RT-PCR analyses using gene-specific primers. As a reference for equal loading the 18SrRNA was used. Almost all of the analyzed plastid genes, regardless of whether they were transcribed by the plastid-encoded (PEP) or the nuclear-encoded (NEP) polymerase, gave rise to similar mRNA levels in *sco2* and wild type (Fig. 3a). The PEP-dependent psbK expression was reduced in 3 day-old seedlings (0.64 ± 0.08 compared to WT). Minor decreases in

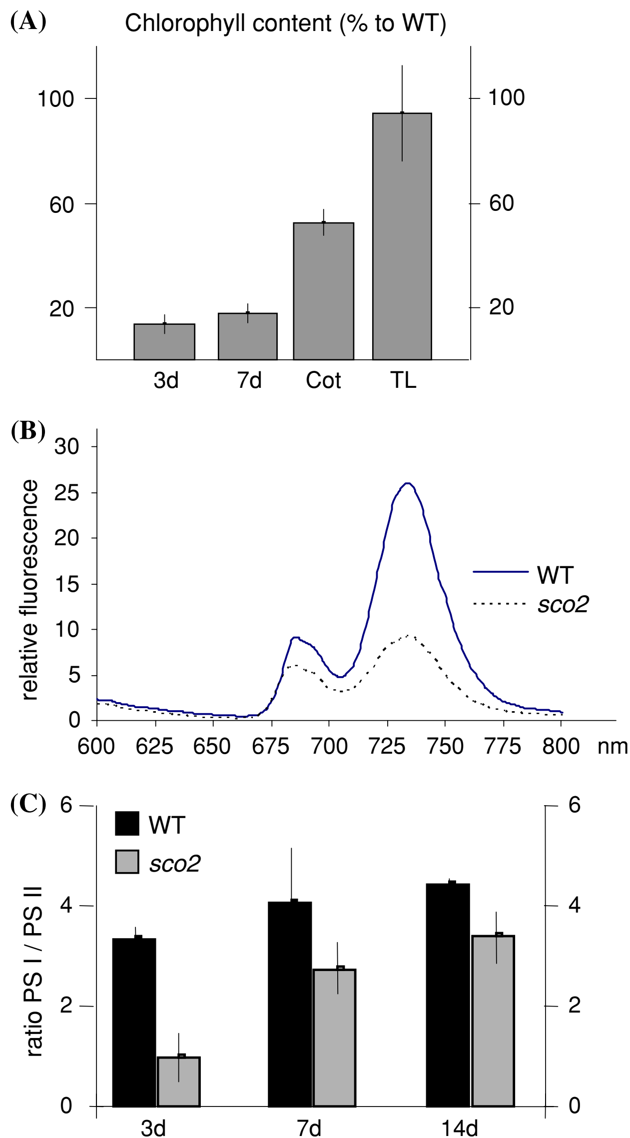

Fig. 2 The chlorophyll content and the accumulation of photosystems I and II in *sco2* and wild type. (**a**) The chlorophyll content has been determined in 3 day-old (3d) and 7 day-old (7d) seedlings and cotyledons (Cot) and true leaves (TL) of 14 day-old plants of the *sco2* mutant as percent of the chlorophyll content of wild type. (**b**) The assembly of the photosystems PS I (730 nm) and PS II (680 nm) has been monitored by recording the 77 K fluorescence emission spectra of 3 day-old seedlings of *sco2* (dotted line) and wild type (solid line). (**c**) The ratio of the 77 K fluorescence emission peaks of PS I to PS II at 730 and 680 nm, respectively, of 3 day-old and 7 day-old seedlings and cotyledons of 14 day-old plants of *sco2* (grey column) and wild type (black column). WT, wild type.

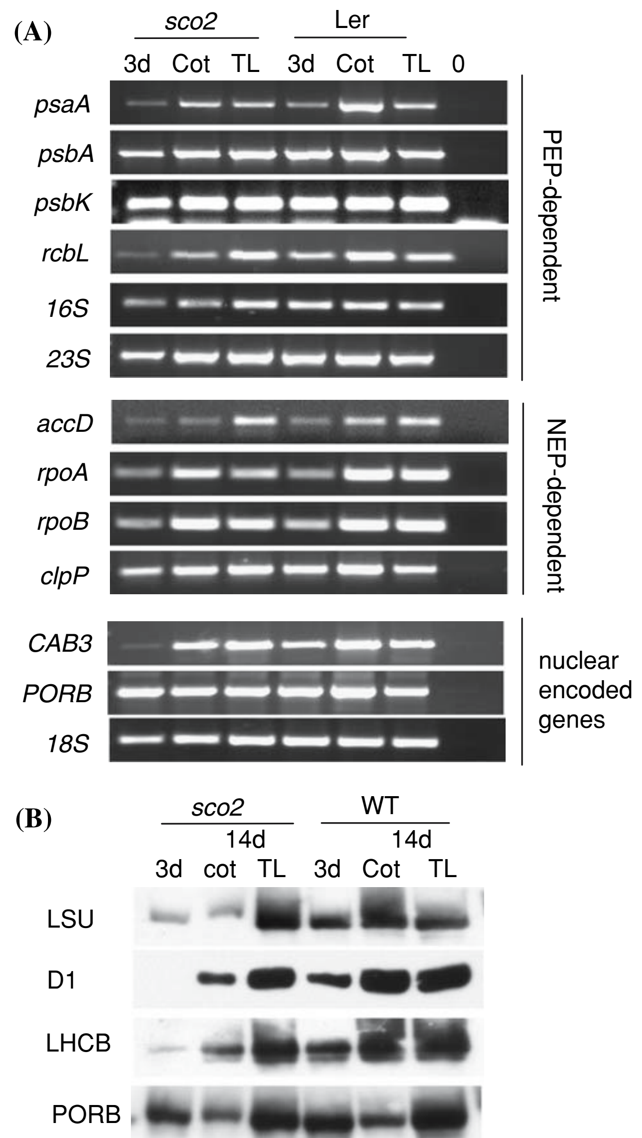

Fig. 3 Gene expression (**a**) and protein accumulation (**b**) in *sco2* and wild type. (**a**) Semiquantitative determination of transcript levels of different plastid and nuclear genes encoding plastid proteins. Total RNA was isolated from 3 day-old seedlings (3d) and cotyledons (Cot) and true leaves (TL) of 14 day-old plants of *sco2* and wild type and was reverse transcribed after DNase treatment. RT-PCR was performed with cDNA-specific primers using 18SrRNA (18S) as a loading control. (**b**) The immunodetection of the chloroplast proteins large subunit of Rubisco (LSU), D1, LHCB, and POR B with monospecific antisera. Equal amounts of protein isolated from 3 day-old seedlings and cotyledons and true leaves of 14 day-old plants of *sco2* and wild type were separated electrophoretically on a SDS gel and transferred onto membranes.

expression compared to WT were observed in the NEP-dependent *accD* expression of 3 day-old seedlings (0.88 ± 0.18) and in *rpoA* in young seedlings and cotyledons (0.88 ± 0.19 resp. 0.88 ± 0.05). Transcript levels of the nuclear *CAB3* gene differed greatly between the mutant and wild type.

Even though the mRNA level of plastid genes was hardly affected by the *sco2* mutation, the accumulation of plastid proteins was reduced significantly in the *sco2* mutant. The levels of different plastid proteins of *sco2* and wild type were determined by using protein-specific antisera. For each sample 25 μg of total protein extract was

loaded onto an SDS gel, separated electrophoretically and subsequently blotted onto membranes. In 3 day-old seedlings of *sco2* the D1 protein was not detectable, whereas the large subunit of rubisco as well as the nuclear-encoded LHCB protein were present in reduced amounts compared to wild type (Fig. 3b). The protein levels in cotyledons increased with increasing age of the seedlings. In true leaves these proteins in the *sco2* mutant reached the same level as in wild type. Among the various proteins analyzed the level of POR B was unaffected by the *sco2* mutation and thus this protein could be used as a loading control. In *sco2* not only the accumulation of membrane proteins such as D1 and LHCB was reduced, but also of soluble stroma proteins such as rubisco. Collectively, these results suggest that *sco2* impacts chloroplast development in cotyledons at the posttranscriptional level.

The embryo development is normal in the *sco2* mutant

Cotyledons are formed already during embryo development and hence are referred to as embryonic leaves. It has been shown previously that during early embryo development chloroplasts are formed transiently and transformed into storage organelles shortly before the onset of seed dormancy (Borisjuk et al. 2005). To investigate if chloroplast development in *sco2* in cotyledons is affected already during embryo development, mature embryos were isolated from siliques of *sco2* and wildtype plants. The isolated embryos of both plants were identical in shape and color (Fig. 4a) and accumulated the same amounts of chlorophyll (Fig. 4b).

Chloroplast development would be expected to proceed during precocious germination of isolated immature seeds of both plants. Isolated immature seeds were taken from the siliques and transferred onto MS media. Germinating premature seedlings of *sco2* developed cotyledons just like wild type and chloroplast development was not impaired (Fig. 4c). Hence *sco2* impedes chloroplast development only in germinating seeds after dormancy.

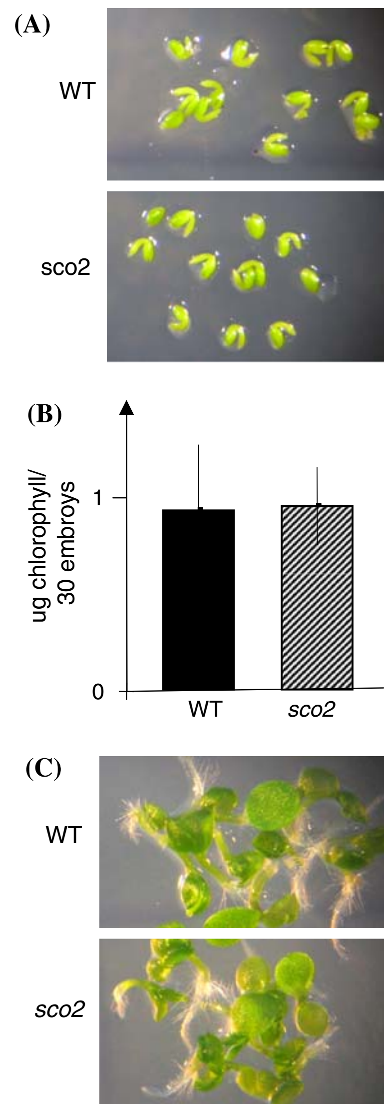

Fig. 4 The *sco2* mutation does not impede chloroplast development in embryos prior to seed dormancy. (**a**) Mature embryos isolated from siliques of the *sco2* mutant and wild type prior to the onset of dormancy. (**b**) Chlorophyll content of mature embryos isolated from the *sco2* mutant and wild type, respectively. (**c**) Precocious germination of isolated mature embryos on MS media.

Seedlings of the *sco2* mutant are not able to cope with an extended period of etiolation

In *sco2* chloroplast development is affected while seedlings undergo the transition from heterotrophic to autotrophic growth. The possible impact of the mutation on the viability of seedlings was analyzed in the following experiment. Seeds of *sco2*, plated on MS agar plates, were retained for two days in the cold, transferred for one hour to light to induce germination, and then kept between 3 and 8 days in the dark before exposing them to continuous light. One week later the survival rate of seedlings was determined by counting seedlings with opened cotyledons and emerging true leaves. The *sco2* mutation had a strong impact on the survival of seedlings. After three days in the dark most of the *sco2* seedlings underwent photomorphogenesis when exposed to light and developed true leaves (Fig. 5b). However, after four days in the dark only 60% and after 5 days in the dark none of the mutant seedlings were able to continue their development after the extended dark period (Fig. 5a, b). Wildtype seedlings, on the other hand, were more robust. Even after 8 days in the dark more than 70 % of the seedlings were able to form chloroplasts

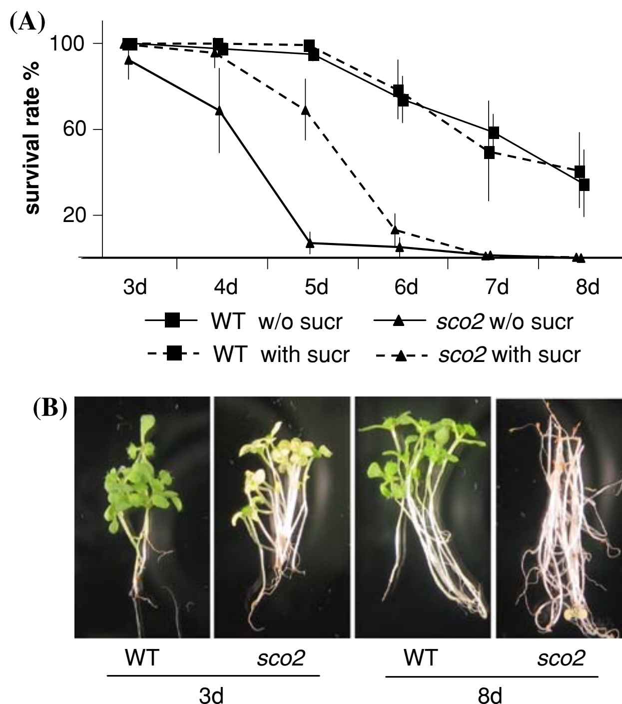

Fig. 5 The effect of an extended dark period on the survival of *sco2* and wildtype seedlings upon illumination. Seedlings were grown in the dark on MS media either with or without sucrose for up to 8 days before being transferred to continuous light. (**a**) After one week in the light the survival rate was determined. (**b**) Phenotype of seedlings that were initially kept for 3 days or 8 days in the dark before being transferred to the light for 7 days.

and continue to green during the following illumination (Fig. 5b). Despite the arrest in photomorphogenesis, mutant seedlings kept for 8 days in the dark and exposure for 7 days to the light do not show cell death as revealed by the lack of lactophenol trypanblue staining of the seedlings (data not shown).

In cotyledons of etiolated seedlings storage materials are provided to support growth in the absence of photosynthesis. The reduced viability of etiolated *sco2* seedlings may be due to a depletion of storage compounds. When *sco2* and wildtype seedlings were kept on a sucrose-containing medium the addition of sucrose enhanced the survival range of etiolated mutant seedlings by one day (Fig. 5a), but after six days of darkness the development of all mutant seedlings was arrested despite the presence of sucrose whereas more than 70 % of the etiolated wildtype seedlings underwent photomorphogenesis in the light.

These results suggest that during the first days after germination seedlings exposed to light require fully active chloroplasts. An impairment of chloroplast activity during this initial developmental phase has a strong negative impact on the seedlings' vitality.

Identification of the sco2 mutation

A map-based cloning approach has been used to identify the mutated gene that causes the impairment of chloroplast development in cotyledons of *sco2*. Homozygous *sco2* (Ler) mutants were crossed with wild type (Col). In the F2 mapping population of this cross 450 recombinant plants were selected to narrow down the chromosomal localization of the mutation to a region of 250 kb on the upper arm of chromosome III between the markers Arlim15 and mi289 (Fig. 6a). Attempts to identify recombination events closer to the *sco2* locus and to find additional polymorphic markers in the vicinity of the mutated gene failed. Hence, a different approach had to be used to identify the *SCO2* gene in the 250 kb fragment. Five BAC clones covering this region were predicted to encode 62 genes. Among these, 11 genes were predicted to encode chloroplast proteins. Because of the pale green phenotype of *sco2* that is likely to be due to an impairment of chloroplast activity these genes were considered to be prime candidates for the *sco2* mutation. However, none of the eleven genes exhibited a mutation. Since predicted mitochondrial proteins may turn out to be localized in chloroplasts, also genes encoding putative mitochondrial proteins were included in the sequence analysis. One of the genes, At3g19220 on BAC MVI11, was shown to be mutated in *sco2-1* at position 124 after the predicted start codon. The exchange of C to T led to a premature STOP codon at the amino acid position 42 (Fig. 6b). In the allelic *sco2-2* mutant a C to T exchange at position 7 was found that led also to a premature STOP codon.

The open reading frame of At3g19220 consists of three exons resulting in a transcript of 564 nucleotides (Fig. 6b). The predicted cDNA encodes an unknown protein of 187 amino acids. To verify if the mutation leads to the loss of mRNA in the mutant lines cDNA-specific primers were designed to amplify a DNA fragment of the gene that was predicted to bridge an intron. Total RNA isolated from 3 day-old seedlings was reverse transcribed and the amounts of the *SCO2* transcript were determined by RT-PCR analysis. With cDNA-specific-primers bridging the first intron (primer 1 and 2) PCR products of the same size could be observed in WT and *sco2-2* mutant. In the *sco2-1* mutant no band was apparent (Fig. 6c). Since the mutation in *sco2-2* mutant is localized just behind the start codon the transcription may start at a second downstream starting point leading to a slightly shorter fragment of the *SCO2* mRNA. To confirm this hypothesis an additional RT-PCR analysis was performed using genomic DNA primers from the start to the stop codon (primer 3 and 4). Using this primer pair no product for the *SCO2* transcript could be amplified in the two mutant lines. Hence, both *sco2* mutants seem to represent knock-out alleles of the At3g19220 gene.

The identification of the *SCO2* genes was confirmed by complementing the mutant with a wild-type copy of the *SCO2* gene. The cDNA of *SCO2* was cloned into the plant binary vector pCAMBIA1302 under the control of the constitutive CaMV 35S promoter and transferred into *sco2-1* mutant plants. The transformed *sco2* mutant plants were comparable to wild type with normal green cotyledons (Fig. 6d).

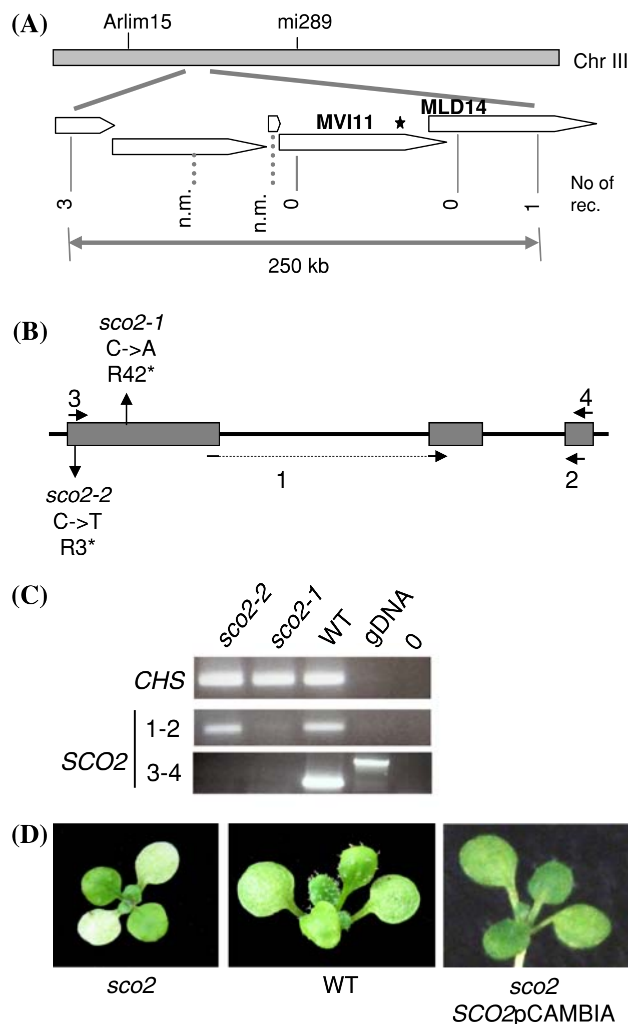

Fig. 6 The identification of the *SCO2* gene. (**a**) The mapping of *SCO2* on the upper arm of chromosome III and BAC clones covering this region. The BAC clone MVI11 containing *SCO2* is marked with an asterisk. (**b**) Localization of *sco2-1* and *sco2-2* mutations within the SCO2 gene and the positions of primers used for the amplification of *SCO2*-specific DNA fragments. (**c**) RT-PCR of *SCO2* transcripts with cDNA- (1 and 2) and genomic DNA primers (3 and 4, encompassing the START and the STOP codon) using DNase-treated total RNA isolated from 3 day-old seedlings of *sco2-1, sco2-2*, and wild type. Genomic DNA was taken as a control to ensure cDNA-specificity. As a loading control cDNA-specific primers for the chalcone synthase gene (CHS) were used. (**d**) Complementation of the *sco2-1* mutant. The cDNA of the wild type *SCO2* gene was cloned in a binary plant transformation vector and used for complementation of the *sco2-1* mutant. WT, wild type; nm, predicted but not confirmed to be a marker.

Localization and expression of the SCO2 protein

According to the databases the *SCO2* gene is predicted to encode an unknown protein with a putative mitochondrial (TargetP, Predotar, and Mitoprot) or nuclear (PSORT and DBSubLoc) localization. The SCO2::GFP fusion protein used for the complementation assay was also taken for localizing the protein within the cell. The green fluorescence of the SCO2 fusion protein co-localized with the red autofluorescence of chlorophyll (Fig. 7a). Hence, the SCO2 protein seems to be localized in chloroplasts rather than, as predicted, in mitochondria or nucleus.

Since the *sco2* mutation leads to a phenotypic change restricted to cotyledons the expression pattern of this gene was determined in different organs of the plant. Total RNA was isolated from seedlings grown for 3 days either in the light or in the dark (etiolated), and from different parts of 6 week-old plants grown on soil that include roots, rosette leaves, cauline leaves, senescing leaves, stem, inflorescence, and young siliques. Reverse transcribed RNA was monitored for the presence of *SCO2* transcripts, using 18SrRNA as a loading control. In all green tissues and to a lower extent also in roots of light-grown plants but not in etiolated seedlings and in

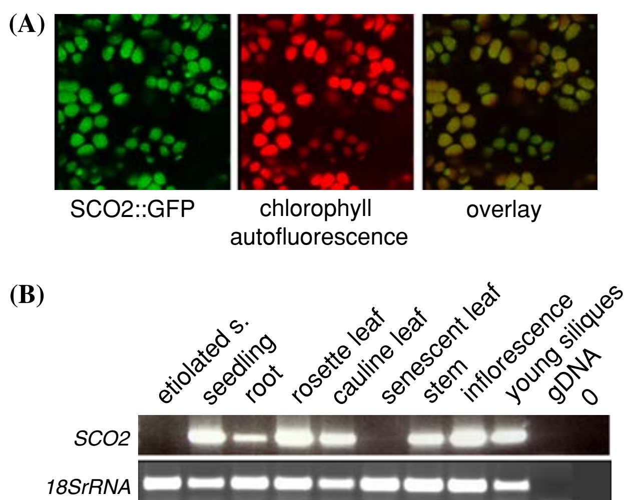

Fig. 7 The intracellular localization of SCO2 (**a**) and expression of *SCO2* in different parts of the plant (**b**). (**a**) The SCO2 protein fused to the green fluorescent protein GFP was stably expressed in plants and detected by confocal laser scanning microscopy. The fluorescence of the GFP fusion protein colocalizes with the autofluorescence of chlorophyll. (**b**) Total RNA of 3 day-old seedlings grown in the dark (etiolated) or in the light, and from different tissues of six-week-old plants was isolated and after DNase-treatment reverse transcribed. PCR was performed with cDNA-specific primers of *SCO2* and of 18SrRNA as a loading control.

senescing tissues the *SCO2* transcript could be detected (Fig. 7b). Hence, the presence of the *SCO2* transcript is not restricted to cotyledons.

Analyses of the AtSCO2 protein

The *SCO2* gene encodes a protein of unknown function. A small region of SCO2 could be identified that shows a 43 % sequence identity with a DnaJ-related protein encoded by a gene localized on chromosome I of *Arabidopsis thaliana* (At1g75690). Closer inspection revealed that this region belongs to a zinc finger domain common for a subgroup of DnaJ proteins. The relationship between the AtSCO2 protein and DnaJ protein was studied further by performing a ClustalW analysis using AtSCO2 and the EcDnaJ protein sequence of *Escherichia coli* (COG0484, Fig. 8a). In addition to some other amino acids the cysteines and glycines of the zinc finger domain are at the same positions in AtSCO2 and EcDnaJ. The complete C-terminus of the DnaJ protein of *E.coli* is missing in the AtSCO2 protein. Also only one of the two zinc-finger domains of the DnaJ protein is present in AtSCO2. Even though the AtSCO2 protein might display a DnaJ-like function it is not a DnaJ protein.

No other AtSCO2-like proteins could be identified in *Arabidopsis thaliana* suggesting that *SCO2* is a single copy gene. In other plant species several SCO2-like proteins could be identified. The alignment of the AtSCO2 protein with SCO2 proteins of *Medicago truncatula* (ABE80527.1) and *Oryza sativa* (Os09g0458400) revealed a highly conserved C-terminus containing the single zinc finger domain (Fig. 8b). Since these proteins were only found among plant species the SCO2 protein seems to be plant-specific.

The *Escherichia coli* DnaJ proteins are involved in protein folding, protein transport or in the degradation of misfolded proteins (Cheetham and Caplan 1998). The highly conserved CxxC domain present in the SCO2

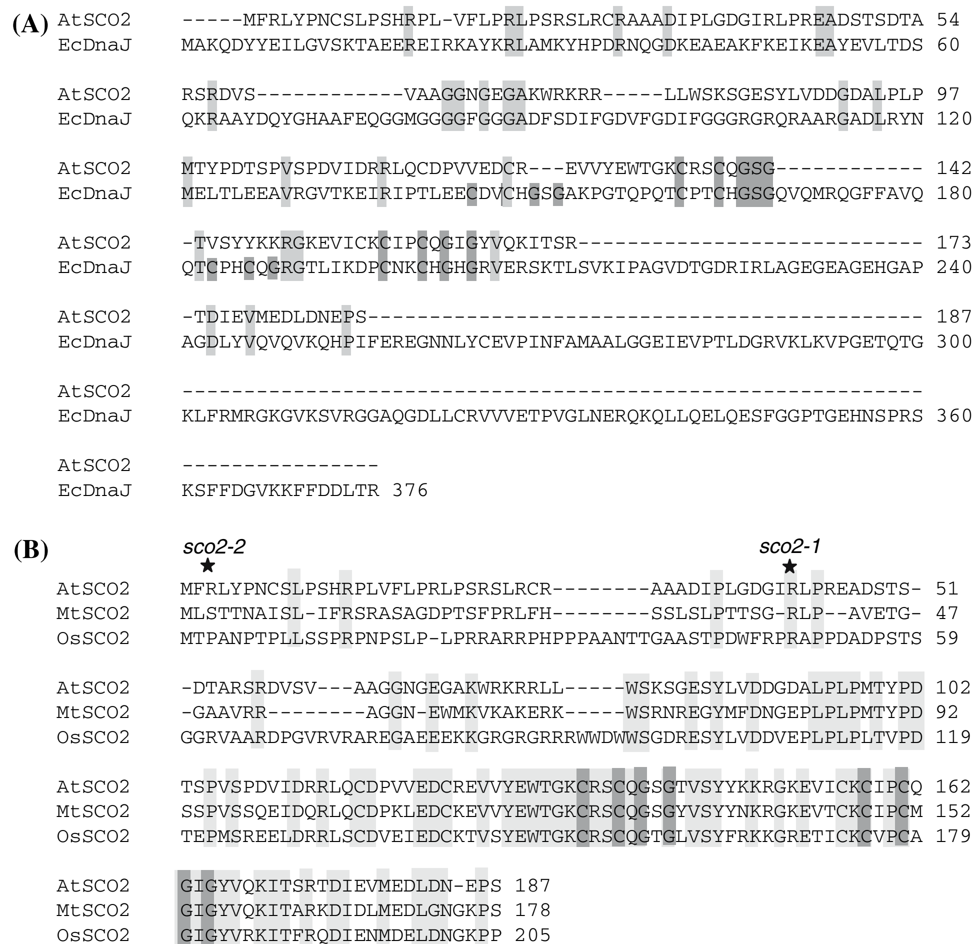

Fig. 8 A comparison of the AtSCO2 protein with (**a**) EcDnaJ (COG0484) and (**b**) different plant SCO2 proteins from *Medicago truncatula* (ABE80527.1) and *Oryza sativa* (Os09g0458400). Conserved amino acids are highlighted with grey boxes and the conserved amino acids of the zinc finger domain with dark grey boxes. Localization of the mutations in the two allelic *sco2* mutant lines are marked with an asterisk.

proteins is also found in protein disulphide isomerases of the thioredoxin superfamily which are involved in protein folding as well (Miernyk 1999). The activity of protein disulphide isomerases can be assayed e.g. by reconstitution of the function of reduced RNaseA (Lyles and Gilbert 1991). The cDNA of SCO2 has been cloned in the Gateway expression vector pHGGWA but no recombinant protein could be purified.

Discussion

Chloroplast development in cotyledons is required for the transition from heterotrophic to autotrophic growth in developing seedlings. Here we describe the characterization of the *snowy cotyledon 2* mutant which suffers from a cotyledon-specific impairment of chloroplast biogenesis.

In recent years several mutants with a defect in chloroplast biogenesis have been isolated including *albino*, *xantha*, and *yellow green* mutants (Chou et al. 2003; Runge et al. 1995; Sundberg et al. 1997). These mutants display an overall pale or white phenotype of the entire seedlings in contrast to *sco2* in which chlorophyll deficiency is restricted only to cotyledons. The former mutants exhibit their pale phenotype already during embryogenesis. In wild type green chloroplasts are formed transiently during embryo development and loose their photosynthetic capacity shortly before dormancy. *albino* and *xantha* mutants do not turn green during embryogenesis (Runge et al. 1995; Sundberg et al. 1997). In contrast, embryos of the *sco2* mutant were fully green prior to the onset of dormancy. Precocious germination resulted in mutant seedlings with wildtype-like green cotyledons. Hence the impairment of plastid function in cotyledons of *sco2* occurs only during germination after dormancy. The function of chloroplasts during embryogenesis is still not clear. Maturing seeds are enclosed within the siliques and shielded from light by the green tissue of the silique valves. Hence chloroplasts of embryos may receive only extremely low light intensities. The plastids of green embryos are referred to as being photoheterotrophic. They fix only very little CO_2 and their main photosynthetic function seems to be the production of ATP and oxygen (Borisjuk et al. 2003). Photosynthesis in embryos that is required for the deposition of storage material within the maturing seed seems not to be essential for seed maturation since chlorophyll-deficient embryos of *albino* and *xantha* mutants accumulate significant storage material that allows them to germinate (Runge et al. 1995; Sundberg et al. 1997).

During the first phase of germination the embryo grows heterotrophically and depends on the storage compounds retained during embryogenesis. This heterotrophic growth can be extended under prolonged dark conditions. *sco2* seedlings kept in the dark loose much faster the ability to change upon illumination from heterotrophic to autotrophic growth than wild type. Although etiolated mutant seedlings kept in the dark for more than 5 days do not continue to grow and green during the following 7 days in the light and seemed to be dead, cell death reactions indicated by positive lactophenol trypanblue staining could not be detected. In the *sco2* mutant supplementation of sucrose in the medium was only partially able to restore the vitality of the mutant. Addition of sucrose to the growth media has been shown previously to rescue several chloroplast biogenesis mutants that had initially been isolated on a sucrose-free medium (Runge et al. 1995; Yamamoto et al. 2000). Thus, the depletion of storage compounds during the growth in the dark may not be the only factor for the reduced vitality of *sco2*.

Most of the mutants isolated so far with a cotyledon-specific defect in chloroplast biogenesis have been shown to be impaired either in plastid gene transcription (Ishizaki et al. 2005; Privat et al. 2003; Shirano et al. 2000), mRNA maturation (Yamamoto et al. 2000), or protein translation (Albrecht et al. 2006). Recently, at the completion of the work a mutant, dubbed *cyo1*, has been described that is allelic to *sco2* and that has been shown to encode a protein disulfide isomerase (Shimada et al. 2007). Therefore, we can assume that SCO2/CYO1 is involved in protein folding. All the mutations have in common that they affect the expression of genes encoding plastid proteins that are needed for the rapid reorganization of the plastid compartment and the assembly of the photosynthetic machinery. During this critical developmental switch from heterotrophic to autotrophic growth limitations in the supply of such proteins may place the developing seedlings under constraints that impede normal chloroplast development and lead to bleaching of cotyledons. With the AtSCO2/CYO1 protein we describe a plastid protein that seems to be essential for the proper assembly of chloroplasts during seedling development.

Material and methods

Plant material and growth conditions

75,000 pooled ethyl-methylsulfonate (EMS) mutagenized seeds of *Arabidopsis thaliana* (ecotype Landsberg erecta) was screened in the M2 generation on MS plates for mutants exhibiting pale cotyledons, but green true leaves. The isolated *sco2* mutant was crossed with wildtype plants of the Landsberg erecta ecotype to verify the recessive character of the mutation and for the purification of the line. Plants were grown under continuous light at 21°C on soil or surface-sterilized seeds were plated on Murashige-Skoog

(MS) media (Duchefa) without sucrose. Sucrose (0.5%) containing media were only used when indicated. Lactophenol-trypanblue staining was performed as described (op den Camp et al. 2003).

Pigment analyses

To analyze the pigment content of seedlings or cut leaves 30 mg fresh weight was harvested. Samples were taken from 3 day-old and 7 day-old seedlings and from cotyledons and true leaves of 14 day-old plants. Plant material was frozen in liquid nitrogen until the extraction of chlorophyll. The leaf material was ground in 1 ml 80 % acetone with barium carbonate and subsequently centrifuged. The supernatant was used for photometric measurements at 645 and 663 nm, respectively, and the OD values were used for further calculations (Porra 2002). The values obtained for wild type were taken as 100 % and the chlorophyll content of the mutant seedlings expressed as percent of wild type. The same approach was used to analyze the chlorophyll content of mature embryos. For each sample the chlorophyll content of 30 mature embryos was determined.

Transcript and protein analyses

For transcript analysis total mRNA was extracted at the indicated time points. The mRNA was DNase-treated and reverse transcribed with Superscript II according to the manufacturer's protocol (Invitrogen). Equal amounts of cDNA were used for further RT-PCR analyses with gene-specific primers using 18SrRNA as a loading control. Primer information can be obtained upon request. Quantification of the RT-PCR analysis has been performed using the spot density measuring tool of AlphaEaseFC (AlphaInnotech, USA).

For protein analysis the total protein was extracted from seedlings as described (Kim and Apel 2004). For each sample 25 μg of protein was loaded on an SDS gel and separated electrophoretically. Rubisco proteins, LHCB, and the D1 proteins and POR B (Kim and Apel 2004) were immunologically detected and visualized as described previously (Albrecht et al. 2006).

Low temperature (77 K) fluorescence measurements

The assembly of photosystems PS I and PS II was assessed by using the low temperature (77 K) fluorescence emission spectra of frozen samples. The fluorescence emission spectra were measured *in vivo* from 600 to 800 nm using an excitation wavelength of 440 nm (Perkin Elmer; Luminescence Spectrometer LS50). The peak level of the fluorescence of PS I at 730 nm was compared to fluorescence maximum of PS II at 680 nm. The analysis was repeated at least three times.

Isolation of the *sco2* mutation and cloning of the *SCO2* gene

Homozygous *sco2* (Ler) plants were crossed with Columbia ecotype plants and homozygous recombinant F2 plants exhibiting pale cotyledons were used for map-based cloning as described (Albrecht et al. 2006). The cDNA of the identified *SCO2* gene was cloned using primers containing NcoI restriction sites for in-frame cloning of the cDNA into the pCAMBIA1302 vector. The correct insertion of the *SCO2* cDNA into the vector was verified by sequencing. *Agrobacterium tumefasciens* cells, strain C58, were transformed with the verified clone and subsequently used for plant transformation using the floral dip method (Clough and Bent 1998). Transformed plants were selected on MS media containing 0.5 % sucrose and 20mg/l hygromycin. The detection of the SCO2::GFP fusion protein was performed as described (Albrecht et al. 2006).

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