Salt tolerance (STO), a stress-related protein, has a major role in light signalling

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Summary
The salt tolerance protein (STO) of Arabidopsis was identified as a protein conferring salt tolerance to yeast cells. In order to uncover its function, we isolated an STO T-DNA insertion line and generated RNAi and overexpressor Arabidopsis plants. Here we present data on the hypocotyl growth of these lines indicating that STO acts as a negative regulator in phytochrome and blue-light signalling. Transcription analysis of STO uncovered a light and circadian dependent regulation of gene expression, and analysis of light-regulated genes revealed that STO is involved in the regulation of CHS expression during de-etiolation. In addition, we could show that CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) represses the transcription of STO and contributes to the destabilization of the protein in etiolated seedlings. Microscopic analysis revealed that the STO:eGFP fusion protein is located in the nucleus, accumulates in a light-dependent manner, and, in transient transformation assays in onion epidermal cells, co-localizes with COP1 in nuclear and cytoplasmic aggregations. However, the analysis of gain- and loss-of-function STO mutants in the cop1-4 background points towards a COP1-independent role during photomorphogenesis.

Keywords: light signalling, phytochrome, blue light, CONSTITUTIVE PHOTOMORPHOGENESIS 1, B-box, Zn-finger protein.

Introduction
Plants as sessile organisms must cope with, and adapt to, a number of environmental cues during their whole life cycle. However, light may be the most important factor controlling and influencing plant development (Franklin et al., 2005). The well-modulated responses of plants to environmental factors is a consequence of established signalling networks, where different molecules are members of one or more pathways that converge, diverge, and interact according to specific environmental conditions and/or developmental stages (Ludwig et al., 2005; Suzuki et al., 2005; Nakagami et al., 2005). For example, it is well documented for abiotic stress that a coordinated crosstalk amongst drought, cold and high salinity pathways exists (Glombitza et al., 2004; Narusaka et al., 2004; Chinnusamy et al., 2004; Mahajan and Tuteja, 2005); however, much less is known about the interplay between light and other environmental signalling pathways.
Salt tolerance protein (STO) is a B-box type Zn finger protein with sequence similarities to CONSTANS (Putterill et al., 1995; Lagercrantz and Axelsson, 2000; Griffiths et al., 2003). It was first identified through a screening approach using a yeast calcineurin mutant. Thus, yeast null mutants in the catalytic subunit genes (cna1cna2), or in the regulatory subunit gene (cnb1), present a salt sensitive phenotype that can be rescued with STO (Lippuner et al., 1996). Surprisingly, in Arabidopsis plants STO gene expression seems not to be induced by salt treatment (Lippuner et al., 1996; Nagaoka and Takano, 2003), although it has been shown that overexpression enhances root growth tolerance to high salinity (Nagaoka and Takano, 2003). In addition, STO interacts with CEO1/RCD1, an Arabidopsis protein that complements an oxidative stress-sensitive yeast strain (Belles-Boix et al., 2000) and negatively regulates a wide range of stress-related downstream genes (Fujibe et al., 2004). CEO1/RCD1 has been recently identified as a new component in the plant salt-stress response, through the interaction with SOS1 (Katiyar-Agarwal et al., 2006). However, an interaction of STO with CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), a negative regulator of photomorphogenesis in the dark, has also been reported (Holm et al., 2001; Ma et al., 2002).
In an attempt to isolate genes involved in general calcium signalling and regulation in plants, we established a complementation screening analysis using a yeast L-type calcium-channel (CCH1) knock-out mutant. This mutant exhibits the same growth arrest in a medium containing high salt concentrations as yeast calcineurin mutants (Paidhungat and Garrett, 1997). Several proteins from Arabidopsis were able to complement this cch1 salt-sensitive phenotype. Amongst them STO appears not only to increase the growth of the knock-out strain in high-salt medium, but also conferred tolerance to higher salt concentrations in the wild-type yeast strain.

To further investigate the function of the STO protein in plants, we isolated a T-DNA insertion line and generated RNAi and overexpressor Arabidopsis transgenic lines. Herein we show that the expression of STO mRNA is light regulated, and that the protein shares a regulatory function in phytochrome and blue-light signalling pathways. Moreover, the STO:GFP fusion protein is actively degraded in the dark in a COP1-dependent manner, whereas light promotes the accumulation of the protein in the nucleus. Additional data shedding light on STO function independent of COP1 are presented.

**Results**

**STO is involved in R, FR and B light signal transduction**

The screening of the Arabidopsis T-DNA Salk database (Alonso et al., 2003) led to the identification of a line containing the T-DNA insertion in the first intron of the STO gene (SALK_067473). In addition, STO RNAi and overexpressor lines were generated and plants homozygous for the transgene were selected for STO mRNA analysis. The RNAi and T-DNA lines exhibited a drastic reduction, if not absence, of STO mRNA, whereas a high constitutive STO transcript level was observed in the overexpressor lines (Figure 1a).

Bearing in mind the interaction of STO and COP1 shown by a two-hybrid system assay (Holm et al., 2001), and that

![Figure 1. Phenotypic analysis of STO transgenic lines.](image-url)

(a) Transcript levels of STO in 3-week-old wild-type (Col-0) and transgenic lines grown under long-day conditions. Equal RNA loading was verified with an 18S rRNA-specific probe.

(b, c, d) Hypocotyl length of 5-day-old wild type, sto-T-DNA and 35S:STO at different fluence rates of continuous (b) red light, (c) far-red light and (d) blue light. Error bars represent the SD of > 30 plants.

(e) Hypocotyl length of 5-day-old wild-type and STO gain- and loss-of-function transgenic lines in dark, continuous red (Rc; 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and blue (Bc; 1.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) light conditions. Error bars represent the SD of > 30 plants.

(f) Relative cotyledon area of 5-day-old wild-type and STO gain- and loss-of-function transgenic lines in Rc (30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Error bars represent the SE of > 45 plants. **Significant differences (\( P < 0.01 \)) compared with the wild type.
COP1 is also a major regulator of light signal transduction (Ma et al., 2002), we investigated whether STO is also directly involved in light signalling by fluence rate response experiments.

Seeds from homozygous T-DNA, RNAi and overexpressor plants were germinated on filter paper and grown for 5 days at 22°C under continuous red (Rc, 0.042–31 μmol m⁻² sec⁻¹), far-red (FRc, 0.017–0.56 μmol m⁻² sec⁻¹) or blue light (Bc, 0.1–10 μmol m⁻² sec⁻¹).

RNAi and T-DNA lines exhibited a pronounced inhibition of hypocotyl growth compared with the wild type under all the light conditions tested. However, the STO-overexpressing lines, including GFP fusion overexpressors (data not shown), developed longer hypocotyls than the wild type under the above described conditions (Figure 1b–e). These results provide evidence that STO is directly involved in light signalling, and suggest a role as a negative regulator of R, FR and B light-mediated hypocotyl elongation. No obvious differences in hypocotyl length were observed when the different homozygous lines were grown in the dark (Figure 1e). Photomorphogenic mutants typically present reciprocal growth responses of hypocotyl and cotyledon cells induced by light signals (Khanna et al., 2006). We measured the cotyledon area of gain- and loss-of-function transgenic lines grown for 5 days in Rc (30 μmol m⁻² sec⁻¹). Both the T-DNA and the RNAi lines exhibited larger cotyledons than the wild type, whereas the overexpressers presented a reduced cotyledon area, providing further evidence for STO function in phytochrome signalling (Figure 1f).

Expression of STO is controlled by light and the circadian clock

In subsequent experiments the transcription pattern of STO mRNA under different light conditions in wild-type plants was investigated. Five-day-old wild-type seedlings grown in the dark were exposed to Rc (10 μmol m⁻² sec⁻¹), FRc (10 μmol m⁻² sec⁻¹) and Bc (10 μmol m⁻² sec⁻¹) light for 3 h, following which STO transcript levels were analysed by Northern blot. The results showed a significant increase of the transcript abundance under all light conditions tested (Figure S1), as compared with dark conditions. These data show that the transcription of the gene is also light regulated.

To unravel the controlling pathway of the light-dependent induction of STO, mRNA expression was investigated in phyA (phyA-211), phyB (phyB-9) and phyA/phyB double mutant lines during de-etiolation under different light conditions. Five-day-old etiolated seedlings were irradiated for 3 h under the above conditions with Rc or FRc light and the STO expression pattern was analysed by Northern blot.

After R light treatment, induction levels of STO were reduced in the phyA, phyB and the phyA/phyB double mutant, by comparison with the wild-type, revealing a contribution of both photoreceptors in the induction of the gene (Figure S1). Under FR treatment, the expression of STO in the wild type reached similar levels as under R light. Also, a clear induction was observed under these conditions in the phyB mutant, in contrast to the phyA and phyA/phyB double mutant, where the transcript levels of STO were reduced (Figure S1). These results suggest that regulation of STO expression under R and FR light is controlled by phyA and phyB. Similar levels of induction were observed for STO after B light treatment in the wild type, phyA, cry1/cry2 and phot1/phot2 double mutants (Figure S1).

Additional experiments to analyse the level of expression of STO in the dark were performed with the cop1-4 mutant line. In contrast to the basic constitutive level of expression present in the wild type, the cop1-4 mutant clearly exhibited an increased level of transcript (Figure S1), indicating that functional COP1 is required to maintain the low transcript level of STO in etiolated seedlings.

We monitored STO transcript levels in adult plants grown under short-day (8-h light/16-h dark) conditions. STO mRNA was present at the end of the dark period and increased during the light phase. In addition, a decrease of the transcript was observed at the beginning of the dark period (Figure 2a). As many light-regulated genes also follow a circadian regulation (Schaffer et al., 2001), we investigated whether the circadian clock might control the transcription of STO. Genes under control of the circadian clock keep on cycling in the absence of external stimuli (Schoning and Staiger, 2005), therefore experiments under continuous light were performed. Three-week-old plants entrained in 12-h light/dark cycles were transferred to continuous light at the end of the daily dark phase, and samples were taken for RNA analysis at different time points during the subjective day/night cycle. STO followed the same transcriptional regulation pattern as that observed under light/dark conditions, typical of genes regulated by the circadian clock. Downregulation of the gene at the end of the subjective light phase and at the start of the subjective dark period, followed by an upregulation at the end of the subjective dark period and during the subjective light phase, were indicative of a circadian regulation of the gene (Figure 2b).

The expression of CHS is regulated by STO

In order to uncover effects of STO on light-inducible genes, an analysis of the expression of well-characterized light-regulated genes (RBCS1b, CAB3 and CHS) was carried out. For this purpose, wild-type, sto-T-DNA and 35S:STO seedlings were grown for 5 days in the dark and exposed to light treatments of 24 h for R (30 μmol m⁻² sec⁻¹), FR (0.5 μmol m⁻² sec⁻¹) or B (1.5 μmol m⁻² sec⁻¹). The results showed no marked variation of the transcript level of CAB3 and RBCS1b in the transgenic lines in comparison with the wild type. However, in the sto-T-DNA line, for CHS,
significant upregulation of the transcript could be observed under FR and B light treatments (Figure 3a). Also we observed that the T-DNA and RNAi seedlings grown for 5 days under FRc or Bc presented a higher anthocyanin accumulation in the upper part of the hypocotyl (Figure 3b).

Accumulation of STO is light dependent

The subcellular localization of STO was analysed in transgenic lines overexpressing STO:eGFP fusions under the control of the CaMV35S promoter. Five-day-old etiolated seedlings were analysed by fluorescence microscopy immediately after the period of growth in the dark, or after transfer to white light (WL) for several hours. In etiolated seedlings no GFP fluorescence was detected (Figure 4a). However, in the seedlings transferred to the light, GFP-containing nuclei started to appear in the hypocotyls after 1–1.5 h of light exposure, and the number increased during prolonged illumination of up to 5 h. In roots and cotyledons fluorescent nuclei appeared after only 3 h of illumination (Figure 4a). In cotyledons the fluorescence signal decreased after 5 h of illumination; in the root it was still visible after 7 h (Figure 4a). After 24 h of continuous light exposure, the GFP signal was barely detectable. The decay of the fluorescent signal was also analysed after transferring the seedlings to the dark, preceded by different periods of illumination. The plants were exposed to light for 1.5 h and transferred to the dark or kept in the light and analysed 1.5 h later.

Figure 2. Diurnal and circadian regulation of STO expression.
(a) Analysis of STO transcription in 6-week-old plants growing under short-day conditions (8-h light/16-h dark) at different time points during the dark or light phase.
(b) Transcript levels of STO in 3-week-old plants grown under 12-h dark/light cycles and transferred to continuous light. Total RNA samples were taken at different time points during the last dark period or after transfer to continuous light. The subjective light/dark cycle is indicated below. Equal loading of the samples was verified with an 18S RNA probe. The graphs show the STO/18S expression of a representative experiment from at least three repeats.

Figure 3. Expression of light-regulated genes in wild-type, STO-overexpressor and T-DNA transgenic lines.
(a) Transcript levels of CHS, CAB3 and RBCS in 5-day-old dark-grown seedlings of wild-type (wt), sto-T-DNA (sto–) and STO-overexpressor (ox) lines before (D) or after illumination for 3 h with red (R), far-red (FR) or blue (B) light. Equal loading of the samples was verified with an 18S RNA probe. The graphs show the quantification of a representative blot, out of at least three repetitions, as relative expression of the genes/18S compared with the wild-type line.
(b) Representative pictures of 5-day-old wild-type (WT), sto-T-DNA, RNAi and overexpressor lines grown under continuous far-red (FR; 1.35 μmol m⁻² sec⁻¹) or blue (B; 37 μmol m⁻² sec⁻¹) light.
later. Whereas the seedlings kept in the light had an increased number of GFP-containing nuclei, those transferred to the dark did not show any fluorescence. Similar results were obtained when transferring the seedlings to the dark after irradiation for 3 h and examination again 2 h later (data not shown). These results indicate that nuclear accumulation of the STO:eGFP fusion increases with time only if the plants are kept under continuous light.

Western blot analysis was performed to investigate whether the absence of GFP signal in the dark, and during the longer exposure to light, was caused by degradation of the fusion protein or re-localization into a different subcellular compartment. Protein extracts of 5-day-old wild-type plants, a transgenic line overexpressing eGFP and transgenic seedlings overexpressing the STO:eGFP fusion were isolated from dark-grown seedlings, or after exposure to WL for either 4 or 24 h. A band of approximately 60 kDa, corresponding to the STO:eGFP fusion protein, could only be detected using polyclonal antibodies specific for GFP in the sample taken after 4 h of light treatment, indicating that during de-etiolation of the seedlings, light stabilizes the fusion protein during the first hours in the cells (Figure 4b).

Co-localization of STO and COP1 in onion epidermal cells

The interaction between STO and COP1 in yeast two-hybrid assays was previously described by Holm et al., 2001; in order to analyse in which subcellular compartment the interaction between STO and COP1 might take place in vivo, localization of STO and COP1 proteins was analysed in transient expression experiments. Expression vectors containing the STO:CFP and YFP:COP1 translational fusions, under the control of the CaMV35S promoter, were used for either single transfection or co-transfection of onion epidermal cells. Single transient transformation of each construct revealed localization of the STO:CFP fusion protein preferentially to the nucleus, although there was also some GFP fluorescence detectable in the cytosol (Figure 5). The fusion protein appeared in a diffuse distribution without any recognizable structures. By contrast, the YFP:COP1 fusion protein was found in aggregations of different sizes in the cytosol, whereas in the nucleus the YFP fluorescence was limited to speckles of more or less equal size (Figure 5), as described previously (Stacey et al., 1999; Stacey and von Arnim, 1999). However, co-expression of STO:CFP and
YFP:COP1 fusion proteins into onion epidermal cells led to co-localization of both proteins in the same nuclear speckles and cytoplasmic aggregations, indicating that the overexpression of COP1 causes recruitment of STO to the same protein aggregations (Figure 5).

COP1 mediates STO:GFP degradation in the dark

The interaction of STO with COP1, together with the spatial–temporal dynamics of STO localization in the cell, raised the question whether COP1 is responsible for the degradation of the protein. Crosses of cop1-4 with a transgenic line over-expressing the STO:GFP fusion protein were performed, and the F2 generation was analysed after growing the seedlings for 5 days in the dark. The cop1-4 mutants harbouring the STO:GFP transgene exhibited GFP fluorescence in nuclei of roots, hypocotyl and cotyledons cells to different extents, whereas the dark-grown wild-type progeny had no detectable GFP. After exposure to light, approximately 75% of those seedlings presenting a wild-type phenotype accumulated GFP in the cell nuclei, as was previously observed for the parental line (Figure 6). In the case of the siblings exhibiting a cop1-4 phenotype, we did not observe a dramatic change of the GFP accumulation during the light treatment (data not shown). These results indicate that COP1 is responsible for the short life of the protein fusion in the etiolated tissues.

Overexpression of STO partially suppresses the cop1-4 phenotype under red light

In order to analyse the possible genetic interactions between cop1-4 and STO, we performed crosses of the cop1-4 mutant with two different transgenic lines overexpressing STO and analysed the homozygous cop1-4_35S:STO lines. Seeds were germinated and grown for 5 days in dark or under Rc (30 μmol m⁻² sec⁻¹), FRc (0.5 μmol m⁻² sec⁻¹) and Bc (1.5 μmol m⁻² sec⁻¹). Hypocotyl measurements showed that the cop1-4 mutant did not differ in the hypocotyl length from homozygous cop1-4 lines overexpressing STO when grown under FRc or Bc. However, under Rc the seedlings overexpressing STO presented slightly (but significantly)
longer hypocotyls than the cop1-4 mutant (Figure 7a), indicating that STO functions independently or downstream of COP1 in the regulation of the red-light mediated inhibition of hypocotyl elongation. No significant differences were observed amongst the dark-grown seedlings (data not shown).

**STO loss-of-function mutants show enhanced light sensitivity in the cop1-4 background**

To investigate a putative COP1 dependency of the STO loss-of-function effects, crosses between cop1-4 and sto-T-DNA were performed and the F3 generation was analysed. Homozygous cop1-4/sto double mutants were grown for 5 days in the dark or under the above-described light conditions, and the hypocotyl length of the progeny from two different crosses was measured (Figure 7b). A clear reduction of the hypocotyl length was observed under the tested light conditions in the cop1-4/sto double mutant, in comparison with the cop1-4 mutant line. This indicates that the STO loss-of-function phenotype is also visible in the absence of active COP1.

**Discussion**

The STO protein from Arabidopsis thaliana was previously characterized as a protein conferring salt tolerance when ectopically expressed in yeast cells (Lippuner et al., 1996). We isolated STO by complementation of a yeast strain that exhibits salt sensitivity, confirming the data obtained by Lippuner et al. (1996). However, Nagaoka and Takano (2003)
reported that overexpression of STO in Arabidopsis resulted in an improved root growth of the seedlings in a medium containing a high salt concentration. We performed salt tolerance experiments using sto-T-DNA and RNAi lines, but our results did not indicate a prominent role for STO in salt stress, as the STO loss-of-function lines did not display any distinguishable salt tolerance or sensitivity phenotype (data not shown). However, our investigations using Arabidopsis gain- and loss-of-function transgenic lines revealed that STO participates in the light-signalling cascade. Inhibition of hypocotyl elongation during de-etiolation is mediated by different plant photoreceptors (Fankhauser and Casal, 2004), and we could show in fluence-dependent irradiation experiments that the STO transgenic lines were affected in this response. These results indicate a significant role for STO as negative regulator of light-mediated inhibition of hypocotyl elongation. Furthermore, the gain- and loss-of-function alleles provoked coordinated reciprocal growth of hypocotyl and cotyledon cells induced by light, providing further evidence that the phenotypes observed are not a consequence of a general cell growth effect of the mutation, as discussed by Khanna et al. (2006). Interestingly, the loss-of-function mutant of a close STO homologue, STH, also presents a short hypocotyl phenotype under R and FR light, but does not show a cotyledon size phenotype (Khanna et al., 2006). Whether STH has a central regulatory function in light signalling, or is involved in specific hypocotyl responses to light, or more general growth processes, still has to be elucidated. In the dark, no marked differences were observed within the T-DNA, RNAi and overexpressing lines compared with wild type, indicating a lack of function of STO during skotomorphogenesis. In addition, analysis of CHS gene expression in the sto-T-DNA lines revealed that STO is required for accurate regulation of the light-dependent expression of this gene. Regulation of CHS transcription in the T-DNA line was significantly altered after FR and B light induction, revealing STO as a negative regulator of CHS expression in B light and phyA dependent FR signalling.

Our analyses of the expression of STO are in agreement with results of microarray studies (Jiao et al., 2003; Tepperman et al., 2001, 2004, 2006). Normal induction of STO transcription during de-etiolation requires functional phyA and phyB, indicating that STO activity in light signalling is downstream of the photoreceptors. Moreover, under B light conditions, induction of STO gene expression is not exclusively dependent on functional phyA, cry1, cry2, phot1 or phot2, suggesting that different photoreceptors might share overlapping functions for the B light dependent expression of this gene.

The analysis of STO transcription in adult plants revealed that the gene is under the control of the circadian clock. The circadian-regulated STO mRNA pattern is similar to that observed for numerous light-regulated genes that typically function in light. Microarray experiments have shown that the wide majority of genes encoding enzymes of the phenylpropanoid biosynthesis are regulated by the circadian clock to peak before dawn, whereas photosynthesis genes peak near the middle of the day (Harmer et al., 2000). In the present study it was shown that the STO transcript level increases in the late dark phase, and remains elevated during the light phase. Thus, the maximal transcript accumulation of genes of the phenylpropanoid biosynthesis pathway is followed by a maximal STO transcript level. Interestingly, it has been recently shown that transcription of SUPPRESSOR OF PHYA-105 (SPA1), a negative regulator of phyA-mediated light responses in Arabidopsis (Hoecker et al., 1998), is regulated by the circadian clock. SPA1 mRNA levels increase at the end of the subjective night and decrease towards the subjective dusk (Harmer et al., 2000; Ishikawa et al., 2006). Similar to the STO loss-of-function mutants, spa1 mutant seedlings accumulate anthocyanin to higher levels compared with wild type under continuous FR and B light (Hoecker et al., 1998; Yang et al., 2005). Thus, it is tempting to speculate that both proteins might be part of a negative regulatory network controlled by the circadian clock, mediating a fine tuning of light-regulated gene expression and thereby preventing an exaggerated light response.

In the dark, COP1 represses STO transcription. COP1 is a negative regulator of light signalling with an active role during skotomorphogenesis (Yi and Deng, 2005). Microarray experiments showed that expression profiles between wild-type seedlings grown under WL and cop1 mutants grown in the dark are qualitatively very similar (Ma et al., 2002). Regulation of STO expression by COP1 would suggest a role for STO downstream of COP1. Nevertheless, STO does not seem to have a function in skotomorphogenesis but only functions during de-etiolation processes. This is supported by the fact that dark-grown cop1-1 seedlings overexpressing STO exhibit the same hypocotyl length as cop1-1 (data not shown). However, a small but significant difference in the hypocotyl length was observed when grown under Rc, albeit that there was no difference (in the tested conditions) when grown under FRc or Bc. The STO-overexpressing phenotype, observed in a wild-type background for all light conditions, surprisingly only appeared under R light conditions in a cop1-4 mutant background. Interestingly, promotion of photomorphogenesis by COP1 under R light, but not under FR or B light, has been observed using weak cop1 alleles, as well as COP1-overexpression lines (Boccalandro et al., 2004; Khanna et al., 2006; Stacey et al., 1999). These observations led to the suggestion of two models in which COP1 would activate phyB-mediated transcription, or alternatively would mediate degradation of a light-induced negative regulator of phyB signalling (Boccalandro et al., 2004). Our data would strengthen the second hypothesis. Under R light conditions, the STO-overexpression
phenotype can be observed in a cop1-4 mutant background, as STO function is activated through a COP1-independent pathway and therefore displays a negative effect on photomorphogenesis. STO could act as the proposed phyB-induced repressor in the model of Boccalandro et al. (2004). However, to the contrary, in B and FR light, the negative regulatory function of STO seemed to be dependent on functional COP1. Interestingly, the analysis of the cop1-4/sto double mutants revealed the same effect of the STO loss-of-function in the cop1-4 mutant as in the wild type, indicating that STO negatively regulates photomorphogenesis partially, if not completely, independently of COP1.

We analysed the light-dependent subcellular distribution of STO in transgenic lines overexpressing an STO:eGFP fusion protein. Accumulation of the chimeric protein in the nucleus is a light-dependent process, whereas disappearance of the fusion protein occurs independently of the light conditions. Moreover, accumulation of the protein in the nucleus is not a result of subcellular redistribution but of light-dependent stabilization.

We could also show that the degradation of the protein in the dark is mediated by COP1. COP1 is a RING-type E3 ubiquitin ligase that directly interacts with positive regulators of the light signalling to mediate degradation of these proteins. Modulation by regulated proteolysis is likely to be a central theme for controlling the specificity and the magnitude of STO function, as observed for other molecules (Osterlund et al., 2000; Holm et al., 2002; Saijo et al., 2003; Seo et al., 2004; Bauer et al., 2004; Duek et al., 2004; Park et al., 2004; Seo et al., 2004; Jang et al., 2005; Shen et al., 2005; Yang et al., 2005). Likewise with STO transcription, STO protein abundance is tightly controlled by light. The rapid accumulation of the fusion protein under WL illumination, and the subsequent disappearance after prolonged light exposure, suggest that STO might be required for the initial transition from skotomorphogenesis to light-adapted development.

Holm et al. (2001) reported an interaction between COP1 and STO and the homologous protein STH. Our transient expression experiments using STO:CFP and YFP:COP1 translational fusions indicated the presence of both proteins in the nucleus, and in the cytosol with different pattern distributions. Co-expression of the fusions resulted in the co-localization of both proteins in the same aggregates, indicating that overexpressed COP1 retains the STO protein in these aggregates in all subcellular compartments. This, together with the data published by Holm et al. (2001), would suggest a direct interaction between COP1 and STO in living plant cells. Several positive regulators of light signal transduction co-localize with COP1 after transient expression in onion epidermal cells (Ang et al., 1998; Ballesteros et al., 2001; Holm et al., 2002; Seo et al., 2003; Jang et al., 2005; Datta et al., 2006). These proteins are exclusively localized in the nucleus. Accumulation of the proteins in nuclear speckles occurs either independently of co-expressed COP1 (Ballesteros et al., 2001; Jang et al., 2005; Datta et al., 2006), or depends on the presence of co-expressed COP1 (Ang et al., 1998; Holm et al., 2002), as also observed for STO. However, the relevance of the co-localization of STO and COP1 in cytosolic aggregates remains elusive, and opens new perspectives for a possible function of COP1 in the cytosol.

**Experimental procedures**

**Plant material, growth and light conditions**

All mutants (Reed et al., 1993; McNellis et al., 1994; Reed et al., 1994; Mockler et al., 1999) and transgenic lines used in this study were in the Columbia background and were compared with wild-type Col-0 in all analyses. The sto-T-DNA line (SALK_067473) was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). Seeds were placed on filter paper soaked in water and kept for 48 h at 4°C in the dark for stratification. After exposure to WL (100 µmol m⁻² sec⁻¹) for 9 h to stimulate simultaneous germination, seeds were transferred to different light conditions. De-etiolation experiments were performed in continuous R, FR and B light at different fluence rates. The light sources used are described in Kircher et al. (2002) and Kaiser et al. (1995).

**Hypocotyl and cotyledon measurements**

Hypocotyl length of seedlings was measured to the nearest 0.5 mm with a ruler or using Zeiss Axiosvision 4.0 software. The absolute length of at least 30 seedlings per line was estimated and the average value was used for comparison of the different lines. Cotyledon area was measured with the same software package. Statistical analysis was performed as described by Khanna et al., 2006.

**Identification of the sto-T-DNA line**

Database research (http://www.arabidopsis.org) led to the identification of an STO-T-DNA line (Salk_067473) containing the insertion in the first intron of STO. The T-DNA was verified by PCR using the left-border specific primer Lb0_5' - GCGTGGACCGCTTGCTGCA-ACT-3' and an STO-specific reverse primer 5'-GGGAAGCTTGAACAAAACTCAAACAGACATTGT-3'. The specificity of the corresponding PCR product was verified by sequencing.

**Plasmid construction**

STO full-length cDNA was amplified by PCR from an A. thaliana cDNA library (Matchmaker; Clontech, http://www.clontech.com) using 5'-CGGATCCATCATCACCCTACTTGGTCTCCCA-3' as the forward primer and 5'-GGGAAGCTTTGAAACAATCCTAACAGACATTGT-3' as the reverse primer, and was cloned into the plant binary vector pCambia 1390.35S. The STO RNAi plasmid was constructed by PCR amplification of a STO sense and antisense fragment using the following primers: sense-for, 5'-AATCTGAGACCTTGGAGCTTTCACAACACA-3', and sense-rev, 5'-TCGGTACCGGTCTCAACCTGCGCTTC-3'; antisense-for, 5'-AATCTGAACGTAGCTGCTTCACAAACAC-3', and antisense-rev, 5'-TCAAGCTTGGTCTCCAAACCTCGGCTTC-3'. Both fragments were
cloned first into the T-DNA cassette of the *phannilus* vector before introducing it into the plant binary vector pArt27, as described by Wesley *et al.*, 2001; The 3SS:*STO:eGFP* vector was generated by PCR amplification of *STO* using the forward primer, 5'-CGACCGGATCCCTCTGTTCCCAACA-3', and reverse primer, 5'-GAACCGGTATAGCTTGAAGCAGATCGGAGAACA-3'. The product was digested with Age I and cloned into the plant binary vector pEGAD (Cutler *et al.*, 2000).

For the co-localization construct, *STO* cDNA was amplified by PCR using the forward primer, 5'-GGGTGACCATCCACCTACCTTGTTCCCAACA-3', and the reverse primer, 5'-TTTACCCGGGATCAGGACACTGAAGTGGTCC-3'. The PCR fragment was cloned in frame with the *CFP* cDNA in the plasmid pMAV_35S:CFP.

**Plant transformation and selection of transgenic lines**

The plant binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101, and Arabidopsis wild-type plants (Col-0) were transformed via the floral-dip method (Clough and Bent, 1998). For selection of transgenic lines, surface-sterilized seeds were germinated on 0.5 x MS, 1% sucrose, 0.8% agar media supplied with either 25 μg ml⁻¹ hygromycin B or 50 μg ml⁻¹ kanamycin. Plants containing the pEGAD constructs were grown on soil and after 2 weeks were sprayed with Basta (240 μg ml⁻¹, 0.005% Silvet L-77).

**Northern blot analysis**

Total RNA was isolated using Plant Plant RNA Extraction Reagent, according to the manufacturer's protocol (Invitrogen, http://www.invitrogen.com). RNA-blot hybridizations were performed following standard protocols (Sambrook *et al.*, 1989). Specific probes for the different genes were amplified from genomic DNA using the following primer sets: *CAB3*-for, 5'-CTGCGAACCAACTTGGTTC-3'; *CAB3*-rev, 5'-TAGGGTTGATATGGCAGATAATCA-TAC-3'; *CHS*-for, 5'-GTGCCCATAGCCGACATTTGAG-3'; *CHS*-rev, 5'-CACACATCCCTACGTGACTC-3'; *RBCS*-for, 5'-GCACGGGT-TGTACCAGTGAG-3'; *RBCS*-rev, 5'-GGTCCGGATGACAGCTGACAA-3', or were isolated from plasmids containing the *STO* cDNA or the 18S rRNA. Autoradiograms were scanned and quantified using QUANTITTYONE software (Bio-Rad, http://www.biorad.com).

**Transient expression in onion epidermal cells**

Expression was achieved by particle bombardment, performed as described by Klein *et al.*, 1987.

**Epifluorescence microscopy**

Epifluorescence and light microscopy on plant seedlings and epidermal stripes of onion cells was performed with an Axiovision microscope with the appropriate filter settings (Zeiss, http://www.zeiss.com). Pictures were taken with a digital camera system using the AXIOVISION software 4.0 (Zeiss). Photographs were mounted with ADOBE PHOTOSHOP (http://www.adobe.com).

**Protein extraction and Western blotting**

Seeds were grown for 5 days in the dark and exposed to WL (100 μmol m⁻² sec⁻¹) for either 4 or 24 h. Proteins were extracted by homogenizing 100 mg of seedlings in a potter using 250 μl of extraction buffer (100 mM NaH₂PO₄, pH 7.2, 1 mM DTT, 7 mM β-mercaptoethanol, 5 mM α-aminocaproate, 1 mM benzamidine). Samples were centrifuged for 10 min at 20 000 g in a microfuge and the supernatants containing the protein extracts were quantified using Bio-Rad Protein Assay Dye Reagent (Bio-Rad). SDS-PAGE sample buffer (5x) was added to the supernatant containing 50 μg of total protein and samples were heated at 95°C for 5 min. Total proteins were separated on a 12% SDS-PAGE gel and transferred onto ImmobilonTM-P transfer membrane (Millipore, http://www.millipore.com). Immunodetection of eGFP was performed using rabbit polyclonal antibodies raised against the green fluorescent protein or mouse monoclonal plant anti-actin (ATACT8) antibodies (Sigma, http://www.sigmaaldrich.com) as primary antibodies, and a peroxidase-coupled anti-rabbit or anti-mouse antibody (Sigma) as secondary antibodies. Detection of the proteins was performed using the ECL™ Western Blotting Detection Reagents Kit (Amersham, http://www.amersham.com).

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**Supplementary Material**

The following supplementary material is available for this article online:

**Figure S1**. Comparison of *STO* expression in wild type and different mutants under different light conditions. *STO* transcription level of 5-day-old etiolated seedlings (Dark) or irradiated for 3 h with blue (B), red (R) or far-red (FR) light. Equal loading of the samples was verified with an 18S RNA probe. The graphs show the *STO*/*18S* expression relative to that of the wild type from a representative experiment out of at least three repeats.

**References**


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