NtSCP1 from Tobacco Is an Extracellular Serine Carboxypeptidase III That Has an Impact on Cell Elongation\textsuperscript{1[C][W][OA]}

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The leaf extracellular space contains several peptidases, most of which are of unknown function. We isolated cDNAs for two extracellular serine carboxypeptidase III genes from tobacco (\textit{Nicotiana tabacum}), \textit{NtSCP1} and \textit{NtSCP2}, belonging to a phylogenetic clade not yet functionally characterized in plants. \textit{NtSCP1} and \textit{NtSCP2} are orthologs derived from the two ancestors of tobacco. Reverse transcription-polymerase chain reaction analysis showed that \textit{NtSCP1} and \textit{NtSCP2} are expressed in root, stem, leaf, and flower tissues. Expression analysis of the \(\beta\)-glucuronidase reporter gene fused to the \textit{NtSCP1} transcription promoter region confirmed this expression profile. Western blotting of \textit{NtSCP1} and expression of an \textit{NtSCP1}-green fluorescent protein fusion protein showed that the protein is located in the extracellular space of tobacco leaves and culture cells. Purified His-tagged \textit{NtSCP1} had carboxypeptidase activity in vitro. Transgenic tobacco plants overexpressing \textit{NtSCP1}-glucuronidase reporter gene fused to the \textit{NtSCP1} transcription promoter region confirmed this expression profile. Western blotting of \textit{NtSCP1} and expression of an \textit{NtSCP1}-green fluorescent protein fusion protein showed that the protein is located in the extracellular space of tobacco leaves and culture cells. Purified His-tagged \textit{NtSCP1} had carboxypeptidase activity in vitro. Transgenic tobacco plants overexpressing \textit{NtSCP1} showed a reduced flower length due to a decrease in cell size. Etiolated seedlings of these transgenic plants had shorter hypocotyls. These data provide support for a role of an extracellular type III carboxypeptidase in the control of cell elongation.

Serine carboxypeptidases (SCPs), family S10 of the Ser peptidase superfamily, have been identified in many plant species (Rawlings et al., 2010). For instance, more than 50 members have been predicted from the rice (\textit{Oryza sativa}) and Arabidopsis (\textit{Arabidopsis thaliana}) genomes (Fraser et al., 2005; Feng and Xue, 2006; Tripathi and Sowdhamini, 2006). SCPs are exopeptidases that catalyze the hydrolysis of a C-terminal peptide bond in proteins and peptides under acid conditions and have a catalytic triad consisting of three essential amino acids, Ser, Asp, and His, which act, respectively, as a nucleophile, electrophile, and base (Barrett et al., 2004; Schaller, 2004). The SCP family is organized into several clades or subfamilies (I–VI) according to their phylogenetic relationships (Supplemental Fig. S1). Some clades contain only plant proteins, while others also contain proteins from animals and fungi (Fraser et al., 2005; Feng and Xue, 2006; Tripathi and Sowdhamini, 2006; Rawlings et al., 2010).

The enzymatic characterization of SCPs is limited, but some information is available for certain members. For instance, types IV and V are prolylcarboxypeptidases requiring a Pro residue in the penultimate position (Mikola, 1986). The SCP family is also called the SCP-like (SCPL) family, because some have been shown to have acyltransferase activity instead of peptidase activity (Schaller, 2004). For instance, SNG1 and SNG2 (for sinapoylglucose accumulator 1 and 2) catalyze the transacylation of sinapoyl esters using sinapoyl Glc as the donor and maleate (SNG1) or choline (SNG2) as the acceptor (van der Hoorn, 2008). The oat (\textit{Avena sativa}) SCP1 is involved in the synthesis ofavenacins, triterpene glycosides (Mugford et al., 2009). This alternative activity of SCPLs might have evolved at the same time as secondary metabolism in plants, which might explain the large diversity of SCPs (Schaller, 2004).

The phylogenetic classification is supported by the structural data. For instance, both type I and II SCPs have two polypeptide chains formed from a single precursor polypeptide by the excision of a linker peptide of about 50 residues (Doan and Fincher, 1988), as in barley (\textit{Hordeum vulgare}) carboxypeptidase I, which is cleaved into two polypeptide chains linked by disulfide bonds, forming the catalytically active heterodimer. In contrast, type III SCPs are single-polypeptide-chain enzymes.

No physiological role has yet been identified for the products of most SCP genes. However, some SCPs are involved in the remobilization of nitrogen resources during seed germination, wound stress, and leaf senescence and may also be involved in various signaling events important for plant growth and...
RESULTS

cDNA Cloning of \textit{NtSCP1} and \textit{NtSCP2} from Tobacco Leaves

Mass spectrometry (MS) analysis of tobacco leaf intercellular fluid resulted in the identification of tryptic fragments typical of SCPIII proteins (Delannoy et al., 2008). To clone the corresponding cDNA, degenerate SCPIII primers were designed based on the conserved upstream (MFYF/LF/LFESR) and downstream (VHDAGHVMFMDQPK) motifs of plant SCPIII s in the MEROPS peptidase database (Supplemental Fig. S2). These primers allowed the amplification of a leaf SCPIII cDNA fragment of 1,107 bp. The 5’ and 3’ ends were obtained by RACE-PCR, and the full coding region was amplified by reverse transcription (RT)-PCR using specific primers. Two cDNAs coding for closely related isoforms, named \textit{NtSCP1} and \textit{NtSCP2}, were obtained. The predicted amino acid sequences were 502 amino acid residues long and 95% identical (Supplemental Fig. S2). The catalytic triad, Ser, His, and Asp, the conserved SCPIII region, GESYA, and a predicted N-terminal signal peptide were identified. Since tobacco is an amphitetraploid with two ancestors identified as \textit{Nicotiana sylvestris} and \textit{Nicotiana tomentosiformis} (Yukawa et al., 2006), we wondered whether \textit{NtSCP1} and \textit{NtSCP2} might actually be orthologs. Using specific primers for \textit{NtSCP1} and \textit{NtSCP2}, we demonstrated by PCR on genomic DNA isolated from the three species that \textit{NtSCP1} came from \textit{N. sylvestris} while \textit{NtSCP2} came from \textit{N. tomentosiformis} (Fig. 1).

Expression Profile of \textit{NtSCP1} and \textit{NtSCP2}

The expression profile of \textit{NtSCP1} and \textit{NtSCP2} was determined by RT-PCR on cDNA obtained from different plant organs (leaf, root, flower, and stem). As shown in Figure 2, both isoforms were expressed in all of the tested tissues, although the expression of both genes in flowers and that of \textit{NtSCP1} in stems was much lower. No major difference according to the developmental stage was observed in stems, leaves, and flowers.

Figure 1. Phylogenetic origin of \textit{NtSCP1} and \textit{NtSCP2}. PCR (30 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C) was performed starting from 200 ng of genomic DNA from tobacco (Nta), \textit{N. sylvestris} (Ns), or \textit{N. tomentosiformis} (Nto) leaves. \textit{ATP2}, encoding the β-subunit of mitochondrial ATP synthase (Boutry and Chua, 1985), was used as a loading control and amplified using the sense primer 5’-TCTTTGCTGGTGTTGGTGAA-3’ and the antisense primer 5’-TGCCTATCATCATAACCAAA-3’. An approximately 600-bp fragment of \textit{NtSCP1} (sense, 5’-GCAAATGCTGTAGGG-GAAC-3’, and antisense, 5’-CATAAAGTGGAATGTTATCTACATG-3’) or \textit{NtSCP2} (sense, 5’-GCAAATGCTGTAGGGGCTCC-3’, and antisense, 5’-CATAAAGTGGAATGTTATCTACATG-3’) was amplified, and a sample of the amplified DNA was electrophoresed (1% agarose gel) and stained with ethidium bromide. The 600- and 700-bp markers are indicated.
To obtain more insights into the expression of one of the two orthologs, we cloned the region upstream of the \textit{NtSCP1} untranslated region from tobacco genomic DNA by inverted PCR (GenBank accession no. GU734644). A 1,575-bp fragment directly upstream of the \textit{NtSCP1} start codon was obtained and fused to the GUS reporter gene. In six positive lines, GUS expression was found in all organs analyzed (Fig. 3), in agreement with the RT-PCR data. Expression was strong in most cells in the roots, except those in the external layer. In the leaves, GUS activity was identified in the epidermis, mesophyll, and conducting vessels. In the stem, expression was seen in the cortex and pith, while in flowers, expression was mainly in the sepals and petal trichomes.

\textbf{NtSCP1 Is Located in the Extracellular Space}

It is assumed that SCPIIs are located in an acidic compartment (Breedam, 1986; Parrott et al., 2005). A previous proteomics analysis of tobacco leaf intercellular fluid (Delannoy et al., 2008) identified a tryptic peptide that fitted the sequence of \textit{NtSCP1} or \textit{NtSCP2} (Supplemental Fig. S2). To confirm the extracellular localization of \textit{NtSCP1}, we added a C-terminal GFP tag on \textit{NtSCP1} and performed transient expression in \textit{Nicotiana benthamiana} leaves. mCherry was used as a marker for the cytosol. After infection, the plants were kept in the dark for 48 h before imaging to avoid the proteolytic degradation of GFP if the protein was located in lytic vacuoles (Zheng et al., 2005).

As shown in Figure 4A, GFP fluorescence was detected at the interface of adjacent cells and did not colocalize with the cytosolic mCherry, confirming an extracellular localization of \textit{NtSCP1}. Plasmolysis was induced by the addition of 200 mM mannitol to enlarge the apoplastic space where \textit{NtSCP1}-GFP was clearly identified (Fig. 4B).

\textbf{Transgenic Tobacco Plants and BY-2 Cells Expressing \textit{NtSCP1-6-His}}

To examine the possible peptidase activity of \textit{NtSCP1}, expression of this protein was attempted in \textit{Escherichia coli}, but no transformant with the expected plasmid was found (data not shown), indicating the possible toxicity of this gene in \textit{E. coli}. Therefore, we expressed a 6-His-tagged \textit{NtSCP1} in tobacco plants and BY-2 cells. Since SCPs might be synthesized as
preproteins, the N-terminal end of the mature protein was unknown and we added the His tag to the C terminus.

Protein extracts from leaf intercellular fluid were prepared from transgenic plants and analyzed by western blotting using anti-5-His antibodies; results for seven lines are shown in Figure 5. In lines NtSCP1-6-His-1, -2, -3, -5, -6, and -7, two bands (apparent sizes of 57 and 65 kD, similar to the expected size of NtSCP1-6-His) were observed (arrows) that were not present in wild-type tobacco or in a transgenic tobacco line expressing the GUS reporter.

As the culture medium of BY-2 cells is more accessible than the leaf intercellular fluid, transgenic BY-2 lines that express NtSCP1-6-His were obtained for further characterization of this enzyme. As a control, we also expressed an NtSCP1 mutant in which His-478, a residue belonging to the catalytic site, was changed into Ala (NtSCP1-6-His His-478Ala). This mutation introduced in the corresponding residue of BRS1 was shown to inactivate the enzyme (Zhou and Li, 2005). As shown in Figure 6A, two bands (57 and 65 kD) similar to those seen in the leaf sample were identified by western-blot analysis of the extracellular medium of several transgenic lines for both constructs. The extracellular medium from 4- to 7-d-old BY-2 cultures (middle to end of the exponential growth phase) of a wild-type line and two transgenic lines expressing NtSCP1-6-His was analyzed by western blotting (Supplemental Fig. S3). The signals were most intense on culture day 4 for both transgenic lines.

The extracellular medium from a 4-d-old culture of the wild type, an NtSCP1-6-His line, and an NtSCP1-6-His-478Ala line was recovered by filtration, adjusted to pH 8, and submitted to purification on a nickel column. After gel electrophoresis, Coomassie blue staining showed several bands that did not qualitatively differ between wild-type BY2 cells and the lines expressing NtSCP1-6-His or NtSCP1-6-His His-478Ala (Fig. 6B, top panel). These bands probably correspond to His-rich proteins. However, two bands present in each transgenic sample and absent in the wild-type sample were found at the same size as the NtSCP1 bands observed before. This was confirmed by western blotting using anti-5-His (Fig. 6B, bottom panel). When the two bands were retrieved from the gel, digested with trypsin, and analyzed by MS, both gave peptides expected from a trypsin digest of NtSCP1-6-His (Supplemental Fig. S4), suggesting that one undergoes posttranslational modification.

The SCP amino acid sequence does not allow identifying whether a particular enzyme is a true peptidase or an acyltransferase (Schaller, 2004). Therefore, we sought to determine NtSCP1 activity. The activity of SCPIII peptidases can be monitored by the use of an artificial substrate, Z-Ala-Phe. The nickel-purified material from a wild-type line, an NtSCP1-6-His line, and an NtSCP1-6-His His-478Ala line was tested in this assay. A clear activity was observed with the NtSCP1-6-His sample. For both the wild-type line and the NtSCP1-6-His His-478Ala line, a background activity of less than 20% was observed, probably resulting from contaminating proteins copurified by nickel chromatography (Fig. 6C). NtSCP-6-His activity was sensitive to phenylmethylsulfonyl fluoride (PMSF), an inhibitor of Ser peptidases (Fig. 6D). These data show that NtSCP1 is a functional peptidase of the SCPIII. NtSCP1 showed a broad activity profile according to the pH, with an optimum around pH 5.0 (Supplemental Fig. S5), in agreement with the acidic apoplastic pH.
Plants Expressing NtSCP1-6-His Have Shorter Petals

Tobacco lines expressing NtSCP1-6-His displayed a very clear phenotype: the flower length was reduced by 18% to 28% compared with wild-type plants (Fig. 7, A and B). Generally, self-fertilization did not take place spontaneously, as the stamens were shorter than the...
carpel (Fig. 7, A and C), and had to be performed by hand. 

Figure 8. Reduced hypocotyl elongation in the NtSCP1-6-His lines. Seeds of three control lines (C1, C2, and C3) and three lines over-expressing NtSCP1-6-His (lines 1, 2, and 5) were germinated and grown in the dark. The hypocotyl length was measured for 12-d-old seedlings. Data are means ± 95% confidence interval (n = 18). Significance was tested using Student’s t test (* P ≤ 0.001).

but NtSCP2 transcripts were more abundant in stems and flowers than NtSCP1 transcripts. This indicates that these two orthologs, brought together in the amphidiploid tobacco, most probably differ in their promoter sequences, leading to a slightly divergent expression pattern. At this stage, we cannot infer whether the different expression patterns already existed in the ancestor genomes or whether they resulted from mutations that appeared after the two genomes were united in tobacco. Whether these differential expression patterns have functional consequences remains to be investigated. The amino acid variation (5%) between the two encoded proteins is limited to positions at which no fully conserved sequence was found in the alignment of several closely related SCP proteins (Supplemental Fig. S2). Considering that these two genes are orthologs in the parent species, N. sylvestris and N. tomentosiformis, it is unlikely that the differences in amino acid sequence have important functional consequences.

The closest NtSCP1 and NtSCP2 homologs in Arabidopsis (At3g45010, At5g22980, and At3g10410) and rice (OsO2g02320 and Os07g29620) belong to a phylogenetic clade with a limited number of plant genes (Supplemental Fig. S1). They have not been characterized yet. However, fungal SCP genes are found in the same clade, contrary to other and larger plant SCP clades (Fraser et al., 2005; Feng and Xue, 2006). This suggests that NtSCP1, NtSCP2, and their close plant homologs correspond to the most ancestral genes of the carboxypeptidase family.

As it is assumed that SCPIIIIs are located in an acidic compartment (Parrott et al., 2005), we checked the subcellular location of NtSCP1 by fusing it to GFP. After transient expression in N. benthamiana leaves, we demonstrated its location in the extracellular space, an acidic compartment (Fig. 4). This confirms the results
of a previous MS analysis that identified some of the peptides corresponding to the SCPIII sequence in the leaf intercellular fluid (Delannoy et al., 2008) and our own MS results for the extracellular medium of BY-2 cells (data not shown). Moreover, western blotting using anti-His antibodies showed that NtSCP1-6-His was found in the leaf intercellular fluid of transgenic plant lines (Fig. 5). This was confirmed in transgenic tobacco BY-2 cells, which showed the expression of NtSCP1-6-His and NtSCP1-6-His His-478Ala in the extracellular medium (Fig. 6). The few SCPs characterized so far have been found to be located in the vacuole (van der Hoorn, 2008), with the exception of BRS1, belonging to clade II, which was shown to be located in the extracellular medium (Zhou and Li, 2005). Interestingly, AtSCP1-49, an SCPIII-like protein phylogenetically close to NtSCP1, is a vacuolar protein, although, in this case, a possible location in the extracellular medium was not tested (Fraser et al., 2005). This indicates that phylogenetic proximity does not imply a similar subcellular localization.

The bands observed in the western-blotting studies using anti-His antibodies had apparent masses of approximately 57 and 65 kD, and the predicted mass of NtSCP1-6-His (57.3 kD) is in the same range. We cannot be more precise at this stage, as (1) we should subtract the mass corresponding to the predicted signal peptide removed in the endoplasmic reticulum (giving a pre-protein mass of approximately 57 and 65 kD, and the predicted mass of NtSCP1-6-His). The intervention of an extracellular peptidase in cell elongation has been reported for BRS1, an Arabidopsis SCPII peptidase (Li et al., 2001; Zhou and Li, 2005). The roles of BRS1 and NtSCP1, which belong to two different Ser carboxypeptidase families, are likely to be different. BRS1 overexpression has a positive effect on plant growth, only leading to limited plant growth when combined with the weak bri1-5 allele of the plasma membrane brassinosteroid receptor (Li et al., 2001; Zhou and Li, 2005), whereas NtSCP1 overexpression results in reduced cell size in the wild-type background.

In conclusion, we have identified an extracellular Ser carboxypeptidase widely expressed in plant organs. Its overexpression limits cell elongation in flower organs and in hypocotyls, suggesting that protein proteolysis is involved in controlling this process. Identification of the substrate(s) of NtSCP1 will be required to fully understand its precise role.
Flower Protein Extraction

Flowers were frozen in liquid nitrogen and then ground with a mortar and pestle in an equal volume of extraction buffer (100 mM Tris-HCl, pH 8.5, 0.2 mM EDTA, 10 mM dithiothreitol, 0.7% polyvinylpolypyrrolidone [Polyclar AT; Serva], 1 mM PMSF, and a cocktail of peptide phosphatase inhibitors from Sigma [1 μg mL\(^{-1}\) each of leupeptin, pepstatin, aprotinin, antipain, and chymostatin]). The homogenate was cleared by centrifugation (20,800 × g, 30 min, 4°C), and the supernatant was used as the soluble fraction.

Purification of NiSSCP1-6-His Using Nickel-Nitrilotriacetic Acid Agarose

Nickel-nitrilotriacetic acid agarose (Ni-NTA; Qiagen) was used for the purification of the His-tagged protein. The Ni-NTA was equilibrated with 100 mM Tris-HCl, pH 7.5, and 5 mM imidazole, then the sample was mixed with the Ni-NTA for 3 h at 4°C on a rotating wheel and the mixture was loaded onto a 1 mL column of Nitrilotriacetic Acid Agarose (Qiagen) equilibrated with 20 mM imidazole, and bound proteins were eluted five times with 0.2 mL of 250 mM imidazole in 100 mM Tris-HCl, pH 7.5. The five fractions were pooled for further analysis.

SCP III Activity

The SCP III activity test was assayed according to Mikola (1986). The reaction was started by addition of the substrate, 1.62 ml L-β-naphthoflavone in 10% dimethyl sulfoxide, washed with 10 volumes of 100 mM Tris-HCl, pH 7.5, and 20 mM imidazole, and bound proteins were eluted five times with 0.2 mL of 250 mM imidazole in 100 mM Tris-HCl, pH 7.5. The five fractions were pooled for further analysis.

Gel Electrophoresis

Proteins were solubilized in Laemmli buffer (2% SDS, 0.125 mM Tris-HCl, pH 6.8, 10% glycerol, 0.002% bromphenol blue, and 60 mM dithiothreitol). After incubation for 60 min at 30°C, the liberated C-terminal Ala-Phe was measured using 2,4,6-trinitrobenzene sulfonic acid (TNBS) reagent, prepared by mixing 3 volumes of 5% sodium tetraborate with 1 volume of 0.2% TNBS (Sigma-Aldrich; P2297). The TNBS reagent (2 mL) was added to the samples, the mixture was incubated for 1 h at 30°C, and then the samples were acidified by the addition of 1 mL of 1 M acetic acid and incubated for 1 h at 38°C. The released C-terminal amino acids Ala-Phe were measured at 340 nm. A standard curve was produced using standard Ala-Phe (Sigma-Aldrich; A3120).

Western Blotting

Western blotting was performed according to standard procedures using mouse anti-5-His antibodies (Qiagen; 1:60) and peroxidase-coupled anti-mouse IgG antibodies (Sigma-Aldrich; A0168; 1:4,000).

Mini Extraction of Genomic DNA

Leaf material (250 mg) was frozen in liquid nitrogen and ground with a mortar and pestle, then 400 μL of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, and 10 mM β-mercaptoethanol) was added to the frozen leaf powder, followed by 35 μL of 20% SDS. The samples were then vortexed and incubated for 10 min at 65°C, 130 μL of 5 M potassium acetate was added, and the mixture was incubated for 10 min on ice and then centrifuged (20,800 × g, 15 min, 4°C). The supernatant was transferred to a microcentrifuge tube and an equal volume of isopropanol was added, and then the samples were incubated for 16 h at −20°C, followed by a centrifugation (20,800 × g, 15 min, 4°C). The pellet was solubilized in water.

cDNA Preparation

Forty micromoles of oligo(dT) (Sigma) was added to 7 μg of total RNA, the volume was brought to 13.5 μL with water, and then the mixture was incubated for 5 min at 70°C and placed on ice for 5 min. An RT mixture (1 μL of RNase inhibitor [Westbury]), 2.5 μL of 10 mM deoxyribonucleoside triphosphate [Westbury], 5 μL of Moloney murine leukemia virus buffer [Promega], and 1 μL of Moloney murine leukemia virus reverse transcriptase [Promega] was added, and the sample was incubated for 1 h at 42°C and for 5 min at 85°C, placed on ice for 5 min, and stored at −20°C.

Cloning of NtSCP1 and NiSCP2 by RT-PCR

A 1,107-bp sequence fragment was amplified using the degenerate sense primer (5'-ATGGTTATATTTTTTTTTTGGAGYAGR-3') and antisense primer (5'-TTNGGGTGGTCCATGNNACCAT-3'). Ten cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by 20 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C. This amplified sequence was cloned into pGEM-T Easy (Promega), sequenced, and found to be 99.2% identical to EST E3232261 from the tobacco BY-2 database (http://mrg.psc.riken.go.jp/strc/). Starting from the nucleotide sequence near the poly(A) of this EST, we designed an antisense primer (5'-TTTTAGGAAAGAAAATATTCCACTCTC-3'), which, in combination with the sense primer above, allowed amplification of the 3' region of the SCPIII gene using the same conditions as in the first PCR. The overlapping sequences of this clone and that resulting from the first PCR were identical. A dCTP tail was added to the cDNA (10 μM dCTP, 25 μM CoCl\(_2\), 2 μL of recombinant terminal transferase [400 units μL\(^{-1}\); Roche] at 37°C for 15 min). An oligo(dG) sense primer and the antisense primer 5'-CTGGTAAACATATACACTAGTATATTCCATTCTCATTCCATTCTCTTATTCC-3' (sense) and 5'-AAAAAATGCTCTTCTTCTCTCTTACCATCATTCCATTCTTATTCC-3' (antisense) and 5'-AAAAAATGCTCTTCTTCTCTCTTACCATCATTCCATTCTTATTCC-3' (sense). The PCR products were cloned into pGEM-T Easy and sequenced.

Generation of the NtSCP1-6-His Expression Vector

The NtSCP1 coding sequence without the stop codon was amplified from the cloned cDNA by RT-PCR (35 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C). The primers used were NtSCP1-SacI (sense, 5'-GGGCTCTAGGCATTACATCCCATCTCTCTTTT-3'; Roche) and antisense primer 5'-TTTTGGAAAGAAAATATTCCACTCTCATTCCATTCCATTCTCTTATTCC-3' (sense). The PCR product (NtSCP1-6-His) was cloned into pGEM-T Easy and sequenced. The NtSCP1-6-His gene was ligated at the KpnI-Sacl sites of the pBAUX3131-EnzPMA4-GFP::NOS plasmid after removal of a GFP-containing fragment (Haseloff et al., 1997) between the KpnI-Sacl sites (Goderis et al., 2002). This plasmid contains the Nicotiana plumbaginifolia PMA4 transcription promoter fused to two copies of the enhancer from the 35S promoter of the Cauliflower mosaic virus (Navarre et al., 2011) and the NOS terminator. Finally, after l-Scl digestion, the NtSCP1-6-His gene was inserted into the cloning vector pZP-RBCS2-nptII (Goderis et al., 2002).

Generation of the NtSCP1-GFP Expression Vector

The NtSCP1 coding sequence without the stop codon was amplified from the cloned cDNA by RT-PCR (35 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C) using the primers NtSCP1-KpnI (antisense, 5'-GGGCTCTAGGCAATTCCGTTCATGTTAGT-3') and NtSCP1-SacI (sense, 5'-GGGCTCTAGGCATTACATCCCATCTCTCTTTT-3'). The resulting PCR product was cloned into pGEM-T Easy and sequenced. The NtSCP1-GFP gene was transferred into the KpnI site in front of the GFP gene of the pBAUX3131-EnzPMA4-GFP::NOS plasmid (Zhao et al., 1999; Goderis et al., 2002). After l-Scl digestion, the NtSCP1-GFP chimeric gene fusion was inserted into the expression vector pZP-RBCS2-nptII (Goderis et al., 2002).

SCP Involved in Cell Elongation

du Tabac) were grown in a phytotron at 72% humidity with a photoperiod of 12 h of light (196 μmol m\(^{-2}\) s\(^{-1}\) at 25°C and 12 h of darkness at 23°C.
Generation of the His-478Ala Mutation in NtSCP1

The NtSCP1-6-His clone was used as a template for the replacement of His-478 with Ala. A partial sequence of NtSCP1-6-His was amplified in two parts using the following primer pairs that contained the His-478Ala mutation (underlined): forward*, 5'-GCAAGATCTGCTAGGGGAAAC-3' / reverse$, 5'-GAACATATCACCCTCTGCT-3' and forward#, 5'-GATATGCTGGCTGATGTCG-3' / reverse#, 5'-TTCGACCTCTAAGGCGATGTCG-3'. (30 cycles with 30 s of annealing at 60.2°C using the Phire polymerase [Finnzymes]). The two PCR products were then mixed and the whole partial sequence was amplified using forward* and reverse$ (29 cycles with 40 s of annealing at 60.2°C using the Phire polymerase). The resulting PCR product was cloned, sequenced, and used to substitute for the original NtSCP1 sequence with the mutated version by BglII and SacI (stabilized in the primer sequences).

Cloning of the NtSCP1 Upstream Region by Inverted PCR

Genomic DNA (7 μg) was digested with EcoRI for 16 h and column purified (Nucleospin ExtractII). Digested DNA (1 μg) was self-ligated with T4 ligase at 16°C for 16 h and column purified. PCR was performed using 100 ng of DNA and HiFi polymerase in a 30-μL total volume (40 cycles of 40 s at 94°C, 40 s at 58°C, and 3.5 min at 68°C) using the "outward-pointing" antisense (5'-CAGAAGAAACAG-CAGAAGGGG-3') and sense (5'-ATCCCCATACAAATTGCTG-3') primers, and a 1,361-bp fragment encompassing the NtSCP1 upstream region was obtained. This sequence was amplified from genomic DNA by PCR using the antisense (5'-AAGCTTTTTTCCTAAAGATTTTGAAT-3'); the HindIII restriction site is underlined) and sense (5'-CAACGACATATGGATACTA-3') primers and HiFi polymerase (Roche) in a 20-μL volume (28 cycles of 40 s at 94°C, 40 s at 58°C, and 1 min 35 s at 72°C). The PCR product was cloned in pGEM-T Easy and sequenced.

The NtSCP1 upstream region was fused to the GUS reporter gene in pAUX3131 (Goderis et al., 2002) using NotI and HindIII, and the whole cassette Nicotiana plumbaginifolia-GUS-NOS was transferred using Agrobacterium tumefaciens to the plant expression vector pPZP-RCS2-nptII (Goderis et al., 2002).

Agrobacterium tumefaciens Transformation by Electroporation

*A. tumefaciens* transformation was performed according to Mattanovich et al. (1989).

Transient Plant Transformation

Liquid 2YTCS medium (3 mL) containing 1.6% bacto-tryptone, 1% bacto-peptone extract, 0.1% Glc, 0.02% MgSO4, 0.8% agar, 20 μg mL⁻¹ rifampicin, 40 μg mL⁻¹ gentamycin, and 100 μg mL⁻¹ spectinomycin was inoculated with a colony of the *A. tumefaciens* clone, the culture was incubated with agitation for 24 h at 28°C, then the cells were harvested by centrifugation (5,000 × g, 2 min, 18°C), washed three times with infiltration buffer (50 mM MES-HCl, pH 5.6, 0.5% Glc, 2 mM NaPO4, and 100 μM acetoxyrin), and resuspended in infiltration buffer, and the density was adjusted to an optical density at 600 nm of 0.1. *Nicotiana benthamiana* leaves were then infiltrated on the abaxial side through the stomata using a syringe, and the plants were incubated for 48 h at 25°C in the dark.

Stable Plant Transformation

Stable plant transformation was performed according to Horsch et al. (1986).

BY-2 Cell Transformation

BY-2 suspension cells were transformed as described previously (An, 1985).

Leaf Intercellular Fluid

The leaf intercellular fluid was obtained as described by Delannoy et al. (2008).

Hypocotyl Length

For hypocotyl measurements, the seeds were germinated and grown on MS medium supplemented with 100 mg L⁻¹ kanamycin for 12 d at 25°C in the dark. The plates were scanned, and the hypocotyl length was measured using Macbiophotonics ImageJ software. The mean for 18 or more plants was taken, and significance was tested using Student's t test.

Confocal Microscopy

Confocal images were acquired using a Carl Zeiss 710 confocal microscope. GFP was excited with the 488-nm line of the argon multilaser, and mCherry (soluble in the cytosol) was excited with the 543-nm line. The emitted fluorescence was collected between 489 and 569 nm (GFP) and between 585 and 696 nm (mCherry) using the online fingerprinting acquisition mode (Carl Zeiss 710).

GUS Staining

The root, leaf, and flower tissues were fixed by vacuum infiltration in 50 mM phosphate buffer (34.2 mM NaHPO4, 15.8 mM NaH2PO4, and 0.05% Triton X-100, pH 7.2) immediately after the addition of 4% of a 37% formaldehyde stock solution. In the case of the flowers, the buffer also included 62 mM β-mercaptoethanol. After 10 min, the tissues were washed three times for 5 min each with the phosphate buffer and incubated at 37°C in the same buffer containing 50 mM K3[Fe(CN)6]. 35 mM K3[Fe(CN)6] and 100 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid until staining appeared.

For sectioning after GUS staining, the material was incubated at 4°C in increasing concentrations of ethanol (30%, 50%, 70%, 85%, 95%, and 100%), each for 20 min, then at 18°C for 30 min in 100% ethanol, 30 min in a 1:2 LR White resin/ethanol mix (London Resin Company), and 16 h in 1:1 LR White resin/ethanol mix. After polymerization for 16 h at 70°C, 2-μm sections were cut using an ultramicrotome (Ultrrotome III, Ultramicrotome Control Unit Type 8802A; LKB Instrument) and transferred in water onto a microscope slide, dried, and covered with Eukitt quick-hardening mounting medium.

Matrix-Assisted Laser-Desorption Ionization MS

MS/MS spectra were acquired using an Applied Biosystems 4800 MALDI TOF/TOF Analyzer spectrometer using a 200-Hz solid-state laser operating at a wavelength of λ = 355 nm. MS spectra were obtained using a laser intensity of 3,700 and 3,000 laser shots per spot in the mass-to-charge ratio range of 800 to 4,000. MS/MS spectra were obtained by selecting the 20 most intense precursor ions per spot and using a laser intensity of 4,200 and 2,100 laser shots per precursor. The automatically selected precursor was subjected to a collision energy of 1 kV with air as the collision gas at a pressure of about 1 × 10⁻⁹ Torr. Data were collected with Applied Biosystems 4000 Series Explorer software. MS and MS/MS queries were performed using the Applied Biosystems GPM Explorer 3.6 software working with the Matrix Science Mascot Database search engine version 2.1. The National Center for Biotechnology Information database was consulted with a search orientated toward green plants. A precursor tolerance of 200 ppm for MS spectra and a 0.1-D tolerance for MS/MS spectra were allowed. The selected charge state of +1, one trypsin miscleavage, and variable modifications consisting of Met oxidation and acrylamide-modified Cys were used for the search.

Sequence data from this article can be found in the GenBank/EMBL database libraries under accession numbers GU734644, ABQ65859, and ABQ65860.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic analysis of NtSCP1 and NtSCP2 as well as representative SCPs from other species.

Supplemental Figure S2. Sequence alignment of NtSCP1 and NtSCP2 with plant SCPs.

Supplemental Figure S3. Expression of NtSCP1-6-His in the extracellular medium of tobacco BY2 cell lines at different growth stages.
Supplemental Figure S4. MS/MS analysis of nickel-purified NiSCP1-6-His.

Supplemental Figure S5. NiSCP1-6-His activity according to pH.

Supplemental Figure S6. Analysis of the flower phenotype of a segregation population of plants expressing NiSCP1-6-His.

Supplemental Figure S7. Expression of NiSCP1-6-His and NiSCP1-6-His His478Ala and flower phenotype in tobacco.

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