Subtilisin-like serine proteases (SBTs) are extracellular proenzymes that depend on their propeptides for zymogen maturation and activation. The function of propeptides in plant SBTs is poorly understood and was analyzed here for the propeptide of tomato subtilase 3 (SBT3PP). SBT3PP was found to be required as an intramolecular chaperone for zymogen maturation and secretion of SBT3 in vivo. Secretion was impaired in a propeptide-deletion mutant but could be restored by co-expression of the propeptide in trans. SBT3 was inhibited by SBT3PP with a $K_d$ of 74 nM for the enzyme-inhibitor complex. With a melting point of $87^\circ C$, thermal stability of the complex was substantially increased as compared with the free protease suggesting that propeptide binding stabilizes the structure of SBT3. Even closely related propeptides from other plant SBTs could not substitute for SBT3PP as a folding assistant or autoinhibitor, revealing high specificity for the SBT3-SBT3PP interaction. Separation of the chaperone and inhibitor functions of SBT3PP in a domain-swap experiment indicated that they are mediated by different regions of the propeptide and, hence, different modes of interaction with SBT3. Release of active SBT3 from the autoinhibited complex relied on a pH-dependent cleavage of the propeptide at Asn-38 and Asp-54. The remarkable stability of the autoinhibited complex and pH dependence of the secondary cleavage provide means for stringent control of SBT3 activity, to ensure that the active enzyme is not released before it reaches the acidic environment of the trans-Golgi network or its final destination in the cell wall.

SBT3 belongs to the S8 family of serine peptidases (1) and are characterized by a specific arrangement of the aspartate, histidine, and serine residues of the catalytic triad within the active site of the enzyme (2). SBTs include general proteases for relaxed substrate specificity for protein degradation and turnover as well as processing enzymes that cleave selected substrates at highly specific sites (3). Most bacterial SBTs are of the catabolic type including subtilisin E and subtilisin Carlsberg from Bacillus subtilis and Bacillus licheniformis, the prototypical members of the S8A subfamily of subtilases (4). The type-example for subfamily S8B is kexin from Saccharomyces cerevisiae, which was identified as the first SBT from eukaryotes and the first with narrow specificity for dibasic cleavage sites (5). Dibasic cleavage specificity is typically found also in the seven kexin-related proprotein convertases (PCs) in mammals (6). In contrast, all plant SBTs belong to subfamily S8A. They are thus more closely related to bacterial subtilisins than to kexin, but they comprise both, general proteases for protein turnover as well as processing enzymes for limited proteolysis at highly specific sites (7, 8).

Early structural investigations of subtilisin E showed that it is produced with an N-terminal signal peptide targeting the protein to the periplasmic space and a 77-amino acid prodomain, which is located between the signal peptide and the catalytic domain but is not part of the mature enzyme (9, 10). The general proproprotein structure is shared with mammalian and plant SBTs, and it evolved convergently in numerous other serine-, aspartate-, cysteine-, and metalloproteases, highlighting the importance of this type of domain organization (10–14).

Although the catalytic domain is generally well conserved between plant SBTs and the prototypical bacterial SBTs, there are also distinctive plant-specific features. Between the His and the Ser residues of the catalytic triad, most plant SBTs carry a large insertion of some 120 amino acids, the so-called propeptide-associated (PA) domain. X-ray structure analysis of SBT3 from tomato revealed that the PA domain mediates homo-dimerization via interaction with an unusual β-hairpin that is not found in bacterial SBTs resulting in the activation of the tomato enzyme (15). However, structural modeling of representative Arabidopsis SBTs indicated that PA domain-mediated dimerization as an autoregulatory mechanism for enzyme activation is unlikely to be a general property of all plant SBTs (16). Consistent with this notion, dimerization was not observed in the second structurally characterized plant subtilase, cucumisin from melon fruits (17). In cucumisin, the PA domain is located comparatively close to the active site channel and appears to contribute to substrate selectivity (17). Additional distinguishing features of SBT3 and cucumisin as compared with bacterial SBTs include a stabilizing fibronectin III-like domain at the C terminus and the lack of bound calcium (15, 17). Two Ca$^{2+}$ binding sites, one of high and the other of...
Function of Propeptides in Plant Subtilases

FIGURE 1. Alignment of subtilase propeptides. Alignment of the propeptides from six plant subtilases and bacterial subtilisin E, all without their signal peptides and with four N-terminal amino acids of the catalytic domain. Signal peptide cleavage sites were determined with SignalP 4.0 (64). The sequence alignment with secondary structure prediction was generated using PROMALS3D (31) with some manual editing in the loop region between α1 and β2. Part of this loop was exchanged in a domain-swap experiment, and this part is highlighted in white letters on gray shading. Highly conserved residues (identical in >75% of the sequences) and partially conserved residues are shaded in black and gray, respectively. Conserved α-helices and β-stands are indicated as gray helices or arrows above the alignment. Conserved hydrophobic regions N1 and N2 are underlined. Residues are numbered from the first Met. The black arrow shows the autocatalytic processing site at the prodomain junction. Amino acids identified by N-terminal sequencing are underlined in the SBT3PP sequence, and the internal cleavage site residues (Asn-38, Asp-54) are indicated (asterisks).

low affinity, are typically found in S8A subtilases (3), and the binding of calcium ions contributes to enzyme stability (18). Despite the lack of calcium, both SBT3 and cucumisin exhibit remarkable thermal stability indicating that plants evolved different means to stabilize the subtilisin fold (8, 15, 16).

The prodomain was not included in the crystal structure of SBT3 or cucumisin, and its function in plant SBTs is thus still poorly understood. In bacterial proteases, on the other hand, prodomain function has been studied intensively, e.g. in subtilisins and in α-lytic protease, where it is needed for correct folding and enzyme maturation (19, 20). When expressed without their propeptides, these proteases accumulate as partially folded, inactive intermediates that are kinetically trapped in a molten globule-like conformation. In the presence of the corresponding propeptides they acquire their native state indicating that the propeptides assist in protein folding. The function of propeptides has thus been described as that of an intramolecular chaperone in bacterial subtilisins and mammalian PCs alike (21, 22). However, unlike chaperones, which accelerate folding by blocking aggregation or other unproductive side pathways, propeptides act as specific single-turnover catalysts. They reduce the energy barrier of a transition state late in the folding pathway, thus allowing the zymogen to proceed from the molten globule to the native conformation (10, 11, 19, 20, 23, 24).

As the propeptide is not part of the mature enzyme, it has to be processed during zymogen maturation. The process has been studied for bacterial and some mammalian SBTs where it depends on the functional catalytic triad and is thus autocatalytic (25, 26). Also, in tomato SBT3 the prodomain is processed in an autocatalytic and intramolecular reaction, as active-site mutants are no longer processed and cannot be rescued by the wild-type enzyme provided as a separate polypeptide in trans (27). However, autocatalytic processing of subtilisin is not sufficient to release the active enzyme as the propeptide remains non-covalently bound to the catalytic domain acting as an autoinhibitor. Inhibition by the cognate prodomain has been demonstrated for bacterial, mammalian as well as plant SBTs. It likely serves to keep the protease inactive until it reaches its final subcellular destination, thus preventing precocious activation and inappropriate proteolysis (28–30).

This autoinhibitory mechanism implies that the interaction of the propeptide with the catalytic domain and, hence, latency of the zymogen are broken in a compartment-specific manner. In the well studied case of furin, for example, release of the propeptide requires a second autocatalytic cleavage event, which is pH-dependent and thus occurs only when the zymogen reaches the trans-Golgi network (26). How the process might be regulated in plants is entirely unknown. It is also unclear to what extent propeptides of plant SBTs resemble bacterial and mammalian homologs with respect to their role during enzyme folding and which features of the propeptides may contribute to their activity as intramolecular chaperones and enzyme inhibitors. These are the questions that were addressed here for the interaction of tomato SBT3 with its own as well as alien propeptides.

Results

Secondary Structure of the SBT3 Propeptide—To obtain first structural insight into the propeptide (PP) of SBT3 and its relation to the PPs of other plant SBTs and bacterial subtilisin, we generated a structure-based multiple sequence alignment using PROMALS3D, thereby including three-dimensional structure information and secondary structure prediction as additional constraints for the alignment (31). Despite the overall low sequence identity (16.9% between the PPs of SBT3 and subtilisin E), the conserved hydrophobic sequence motifs N1 and N2 that appear to be important for the chaperoning function of previously characterized PPs (21, 32, 33) could also be detected in the plant sequences (Fig. 1). The structural scaffold of two β-α-β motives that is conserved in PPs of bacterial subtilisins and mammalian PCs and important for their interaction with
cognate catalytic domains (21, 34, 35) was also predicted for the PP of SBT3 (SBT3PP) and other plant SBTs (Fig. 1).

To further investigate secondary structure composition, SBT3PP was expressed in *Escherichia coli* as an N-terminally His-tagged fusion protein. As previously reported for the PP of cucumisin (30), SBT3PP was found to be insoluble in *E. coli* and accumulated in inclusion bodies. The recombinant protein was solubilized in 8 M urea and purified to apparent homogeneity by affinity chromatography, and 2 μg were separated by 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. The molecular masses of the marker proteins are indicated.

FIGURE 2. Purification of SBT3 and subtilase propeptides. A and C, subtilase propeptides (A) and 17-kDa, 63-kDa, and 48-kDa bands from the supernatant of a tomato cell suspension culture and, 0.5 μg were separated by 12% SDS-PAGE. B, SBT3 was purified from the supernatant of a tomato cell suspension culture, and 0.5 μg were separated by 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. The molecular masses of the marker proteins are indicated.

To assess whether SBT3PP shows a similar increase in stability after binding to SBT3, we expressed SBT3 in a transgenic cell suspension culture as a homologous expression system and purified the mature recombinant enzyme to apparent homogeneity essentially as described (27) (Fig. 2B). The complex of mature SBT3 with its PP was established at 4 °C with a 10-fold molar excess of the propeptide followed by gel filtration to separate the complex from unbound SBT3PP (Fig. 4). Thermal unfolding of the free protease and the protease-propeptide complex was monitored by CD spectroscopy at 220 nm (Fig. 3B). Consistent with previous reports (15), SBT3 exhibited considerable thermal stability, with 76 °C as the transition point for unfolding. In contrast to bacterial subtilisin, which is destabilized by binding of its propeptide, resulting in a reduced melting temperature of the complex in comparison to the free protease (33), the SBT3-SBT3PP complex showed a marked increase in melting temperature to 87 °C, which was 11 °C above that of free SBT3. Interestingly, unfolding was quicker and the cooperativity of unfolding higher, as indicated by the steeper slope of the transition, for the complex as compared with the free enzyme (Fig. 3B). We conclude that the interaction of SBT3 and its propeptide stabilizes the domain structure of the complex, increasing the already remarkable thermal stability of SBT3 significantly.

Requirement of SBT3PP and Related Plant PPs for Folding and Secretion—The data indicate similarities between the PPs of SBT3 and cucumisin. The latter is the only plant PP characterized so far and was described as an autoinhibitor of its mature enzyme (30). In the following we investigated whether the function of plant PPs goes beyond this inhibitory role and includes that of an intramolecular chaperone for enzyme maturation. According to Shinde and Thomas (21), the chaperone function in the propeptide-mediated maturation pathway of bacterial subtilisins and mammalian PCs is apparent in three distinct stages: (i) the folding of the polypeptide to a structured state, (ii) autoprocessing of the PP resulting in a non-covalently associated PP-inhibited protease complex, and (iii) secondary cleavage of the PP to release the active enzyme. As an additional activity that is closely linked to their role in folding, PPs are also required for secretion or, in eukaryotes, for transport into specific compartments of the secretory pathway (21, 26, 39).

To address the question of whether SBT3 requires folding assistance at all, we denatured the protein and tested whether or not it can refold in the absence of denaturant. Denaturation required harsh conditions because of the exceptional stability of SBT3. In fact, the enzyme retained ~80% activity after incubation in 8 M urea. Upon titration with guanidine hydrochloride (GuHCl), SBT3 unfolded with a transition midpoint at 5.8 M (Fig. 5A). Therefore, to fully denature the protein, it was subjected to 8 M GuHCl or, alternatively, TCA precipitation. In an attempt to renature the protein, TCA/GuHCl was removed by dialysis. Refolding resulted in soluble protein, but differences in...
Function of Propeptides in Plant Subtilases

FIGURE 3. Structure and stability of the SBT3 propeptide. A, circular dichroism spectroscopy of recombinant SBT3PP. SBT3PP (15 μM) was analyzed in 20 mM sodium phosphate buffer, pH 7.5, at 20 °C. Data represent the average of four scans corrected for the buffer control. B, thermal unfolding of SBT3 (closed circles) and the SBT3-SBT3PP complex (open circles). The loss of secondary structure was monitored by CD spectroscopy at 220 nm and is shown as the fraction of protein unfolding with increasing temperature. C, thermal stability of SBT3PP. Thermal unfolding (left) was analyzed in a Prometheus NT.48 instrument (NanoTemper Technologies). Tryptophan fluorescence was recorded at 350 and 330 nm, and the 350/330 nm ratio is plotted against the temperature (black line). There is no well defined peak in the first derivative (dotted line) and, therefore, no clear transition point for protein unfolding (left panel). After the unfolding reaction, protein refolding was recorded in the same assay under decreasing temperature conditions (right panel).

The data suggest that SBT3 requires assistance to complete its folding pathway. However, our attempts to refold the protein in the presence of its PP were not successful and did not result in native-like CD or fluorescence spectra within the timeframe of the experiment (2 weeks). Also in subtilisin, refolding is very slow when the propeptide is added in trans (24). As compared with the intramolecular process, the bimolecular reaction is at least 1000-fold slower, resulting in 50% refolding after 8 days at 4 °C (22).

Being unable to directly demonstrate chaperone activity for SBT3PP in vitro, we looked at the in vivo situation where folding is intimately linked to secretion (39). In eukaryotes, proteins are co-translationally targeted to the endoplasmic reticulum (ER), fold in the ER, and need to pass ER quality control as a prerequisite for further sorting along the secretory pathway and secretion, whereas misfolded proteins are targeted for degradation (40). Secretion was thus used as a proxy for successful folding in vivo (26). Folding/secrecion of SBT3 and its dependence on the PP were analyzed by transient expression of the test proteins in Nicotiana benthamiana plants. We previously used this system to demonstrate that the PP of SBT3 is processed in an intramolecular autocatalytic reaction in the ER (27). Active site (S538A, S538C) mutants of SBT3 accumulated in the ER as unprocessed precursors indicating that the activity of SBT3 and autocatalytic processing are required for secretion of the mature enzyme (27).

The question of whether SBT3 depends on its PP for folding and sorting was addressed by use of a SBT3 deletion mutant (SBT3 ΔPP) lacking the PP sequence (residues Gln-23 to His-112). Upon transient expression in N. benthamiana, wild-type SBT3 was processed and secreted to the apoplastic space. In contrast, SBT3 ΔPP was only detected intracellularly at a much reduced expression level, indicating that the mutant did not pass ER quality control (Fig. 6). Co-expression of SBT3ΔPP with its PP as a separate polypeptide chain partially restored accumulation of SBT3ΔPP and export to the apoplast (Fig. 6). The data confirm a requirement of SBT3 for its prodomain for secretion. They further suggest that SBT3PP, like the PP of subtilisin (19), is able to provide folding assistance in trans. SBT3PP thus resembles PPs in bacterial and mammalian SBTs with respect to their chaperone-like activity (21, 22).

Addressing the question of specificity, additional closely and more distantly related PPs were tested for the ability to substitute for SBT3PP as folding assistants, including the PPs of selected tomato subtilases (SBT1.4, SBT1.2, SBT1.1, and SBT1.3, exhibiting 90, 52, 34, and 34% sequence identity with SBT3PP, respectively) and the closest homolog of SBT3 in Ara-
Function of Propeptides in Plant Subtilases

SBT3 activity. Activity assays contained 90 nM SBT3 and a fluorogenic peptide substrate at 20 μM. The activity of SBT3 was strongly inhibited by its own PP with half-maximal inhibition (IC_{50}) at 184 nM, corresponding to a 2-fold molar excess of the PP over the subtilase (Fig. 7A). Somewhat weaker inhibition of SBT3 activity was also observed for the closely related SBT1.4PP with an IC_{50} of 488 nM, whereas all other tested PPs turned out to be very poor inhibitors of SBT3 (Fig. 7A). ~60% of SBT3 activity was retained even at 100-fold molar excess of the more distantly related PPs (Fig. 7A; SBT1.1PP could not be

FIGURE 4. Purification of the SBT3-SBT3PP complex. SBT3 and the SBT3-SBT3PP complex were separated by gel filtration. Fractions were analyzed by SDS-PAGE and SBT3 activity assay using a fluorogenic peptide substrate. A, gel filtration of SBT3 (100 μg) monitored at 280 nm (solid line). SBT3 activity was assayed in 50 μl aliquots of the fractions (diamonds). 15-μl aliquots of the same fractions were separated by 15% SDS-PAGE, and the Coomassie-stained gels are shown below the chromatogram. The gel on the very left shows the protein preparation used in the experiment containing SBT3 (assayed in 50 μl aliquots of the fractions (diamonds)). 15-μl aliquots of the same fractions were separated by 15% SDS-PAGE, and the Coomassie-stained gels are shown below the chromatogram. 15-μl aliquots of the fractions (diamonds). 15-μl aliquots of the fractions (diamonds). B, SBT3 (46 μg) incubated for 10 min in presence of a 10-fold molar excess of SBT3PP was separated by gel filtration, and fractions were analyzed as described for A. The gel on the very left shows the protein preparations used in the experiment for SBT3 (open and closed triangles) and SBT3PP (asterisk).

FIGURE 5. Structure and stability of SBT3. A, chemical denaturation of SBT3 (1.25 μM) by stepwise addition of GuHCl was recorded by tryptophan fluorescence spectroscopy. The background corrected fluorescence maxima from three independent experiments were plotted against the GuHCl concentration (0–7 M). B, tryptophan fluorescence of native SBT3 (solid line) and SBT3 renatured from 8 M GuHCl (dotted line). C, far-UV CD spectra of native SBT3 (solid line) and SBT3 renatured from TCA precipitation (dotted line) or 8 M GuHCl (dashed line). Samples were incubated in 50 mM HEPES, pH 7.5, containing 50 mM Arg and Glu followed by dialysis against 20 mM phosphate buffer, pH 7.5.
**Function of Propeptides in Plant Subtilases**

SBT3∆PP was expressed in *N. benthamiana* leaves with or without co-expression of different PPs. A, 20 μg of total leaf extract (top) or 4 μg of apoplastic proteins (bottom) were separated by 12% SDS-PAGE. Proteins were visualized by Coomassie-based staining with InstantBlue (Expedeon). The molecular weight of marker proteins is indicated. B, 4 μg of total leaf extract (top) or 1 μg of apoplastic proteins (bottom) from the samples used in A were separated as above and transferred to a nitrocellulose membrane. Blots were developed using a polyclonal antiserum against SBT3 (1:2000; Ref. 27) and a peroxidase-conjugated secondary antibody (1:10000, Calbiochem) with enhanced chemiluminescence detection. The position of SBT3 is indicated (black arrow).

tested in this experiment, because renaturation under different conditions did not result in soluble protein). The interaction of SBT3 with its PP in an autoinhibited complex is thus rather specific; SBT3 does not share the loose propeptide specificity reported for other subtilases, including bacterial subtilisins (41), mammalian PCs (42), and even cucumisin, which was shown to be inhibited by PPs from other plant species (30).

To corroborate these findings we determined the dissociation constant (*Kd*) of the SBT3-propeptide complexes by microscale thermophoresis. Fluorescence-labeled SBT3 (30 nM) was titrated with increasing concentrations of the unlabeled PPs (2.44–5000 nM for SBT3PP; 2.2–9000 nM for SBT1.4PP). Complex formation was recorded as a ligand-dependent change in thermophoresis and was plotted against the PP concentration (Fig. 7B). Dissociation constants of 73.5 ± 10.8 nM and 1120 ± 164 nM were derived from the saturation curves for the interaction of SBT3 with its own PP and with SBT1.4PP, respectively. For the remaining PPs no binding curves could be recorded, indicating that the *Kd* of their interaction with SBT3 is above the detection limit of the thermophoresis assay (>30 μM). A dissociation constant in the two-digit nanomolar range for the SBT3-SBT3PP interaction indicates tight binding of the inhibitory PP to SBT3. A strong interaction between SBT3 and its PP is also consistent with the increase in thermostability that was observed for the complex as compared with the free protease (Fig. 3B). The 15-fold higher *Kd* for SBT1.4PP, the most closely related plant PP, is consistent with its reduced inhibitory potential (3-fold higher IC50PP) and indicates that the interaction of SBT3PP with its cognate protease is highly specific with respect to both formation of an autoinhibited complex (Fig. 7) and folding assistance (Fig. 6).

**FIGURE 7. Interaction of SBT3 with different subtilase PPs.** A, inhibition of SBT3 activity by different PPs. 90 nM concentrations of purified SBT3 were incubated with increasing concentrations of the PPs from SBT3 (closed circles), SBT1.4 (open circles), SBT1.2 (diamonds), SBT1.3 (triangles), or AtSBT1.9 (squares). Activity was recorded over 15 min at room temperature using a fluorogenic peptide substrate. Activity is expressed in percent of SBT3 activity without PPs (100% corresponding to a reaction rate of 427.3 ± 49.2 pmol/min). Half-maximal inhibition (IC50PP) was observed at 158 nM for SBT3PP and at 372 nM for SBT1.4PP, respectively. GraphPad Prism 6 was used for curve fitting (GraphPad software; R2SBT3PP = 0.9715, R2SBT1.4PP = 0.9425). Results represent the mean of at least three independent experiments ± S.D. B, dissociation constants of the SBT3/PP complexes analyzed by microscale thermophoresis. Labeled SBT3 (30 nM) was titrated with unlabeled SBT3PP (closed circles) and SBT1.4PP (open circles). The fraction of SBT3 bound in the complex is plotted against the PP concentration. Data points represent the mean ± S.E. of three biological replicates using independent PP purifications. GraphPad Prism 6 was used for curve fitting (R2SBT3PP = 0.939; R2SBT1.4PP = 0.877).
Aqualysin I as compared with their own PPs, even though there is only 20% sequence conservation (41). The apparent specificity of SBT3 for its own PP also distinguishes it from the plant subtilase cucumisin. Cucumisin is inhibited not only by its own PP but also by PPs from *Arabidopsis* and rice subtilases (ARA12 and RSP1, respectively) that share ~36% sequence identity (30).

Aiming to identify distinguishing features of SBT3PP that may be responsible for the specificity of interaction, we compared the plant PP sequences and found that the loop between helix α1 and strand β2 is extended in SBT3PP and SBT1.4PP, as compared with PPs that lacked inhibitory or chaperone activity (Fig. 1). To test whether this loop extension is responsible for the observed specificity, a domain-swap experiment was performed in which the respective region (residues Ser-57 to Lys-70) was deleted from SBT3PP (SBT3PPΔS-K) and inserted into the shorter loop of the apparently inactive PP of SBT1.3 (SBT1.3PP&S-K; cf. Fig. 1). The mutant PPs were expressed in *E. coli*, purified, and renatured as before (Fig. 2C). The activity of SBT3PPΔS-K as an inhibitor of SBT3 was somewhat reduced as compared with the wild-type PP requiring a 3-fold higher concentration to achieve the same level of SBT3 inhibition (Fig. 8A). The loop extension thus seems to make only a minor contribution to the binding specificity of SBT3PP to the mature protease. It is also not sufficient to promote binding of an otherwise inactive PP, as the inhibitory activity of SBT1.3PP&S-K was not increased as compared with unmodified SBT1.3PP (Fig. 8A).

Chaperoning activity of the modified PPs was analyzed in the transient expression assay for folding/secrection in *N. bentha- miana*. The ability of the SBT3 propeptide to restore folding/secrection was completely lost in the loop-deletion mutant, indicating that the extended loop, in addition to the generally conserved β-α-β-β-α-β fold, is absolutely required for the specific interaction of SBT3PP with SBT3 during zymogen maturation (Fig. 8B). Separation of the two activities, as evident from the complete loss of chaperoning activity as compared with a marginally impaired inhibitory activity, suggests that the PP interacts differently with immature SBT3 during folding and with mature SBT3 in the autoinhibited complex.

**Propeptide Cleavage and Release of Mature SBT3**—The maturation of SBTs requires at least two proteolytic events, the first one separating the PP from the catalytic domain resulting in the non-covalently linked self-inhibited complex and the second one cleaving and destabilizing the PP, which is then released to result in the active enzyme. Both cleavages are autocatalytic in bacterial and mammalian SBTs, and consequently, multiple basic residues are found upstream of the two scissile bonds in PCs, reflecting the substrate specificity of these enzymes (29, 35). Although processing of the prodomain is also autocatalytic in plants (27), the residues downstream of the scissile bond appear to be at least as important for cleavage site recognition. These residues mark the N terminus of the mature proteases, which is highly conserved in plant SBTs (43, 44) with Thr-Thr-Xaa-Thr/Ser as the first four amino acids (Fig. 1; Xaa representing a positively charged or hydrophobic residue). The conserved N terminus suggests that plant SBTs may share a common mechanism for processing site recognition, which appears to be different from that in PCs or bacterial subtilisins.

Addressing the question of whether the internal cleavage of the PP is also autocatalytic in plant SBTs, we assessed the stability of the PP in the purified SBT3-SBT3PP complex by SDS-PAGE (Fig. 9). The complex was found to be stable at neutral pH. However, upon incubation at acidic pH, PP cleavage was observed, first at pH 5.7 and to a stronger extent at pH 5.2 (Fig. 9A). pH-dependent cleavage and degradation of the propeptide was also reported for some mammalian PCs and has been well studied in furin, where it provides a mechanism for compartment-specific activation of the protease (26, 29). Similarly in SBT3, pH dependence of PP cleavage may prevent precocious activation and ensure that the active enzyme is not released before the pH drops along the secretory pathway from pH 6.3 in *trans*-Golgi cisternae to pH 5.6 in the *trans*-Golgi network/early endosome (45).

A time-course of PP cleavage indicated that auto-processing at pH 5.2 is very slow. Significant degradation was observed only after overnight incubation (Fig. 9B). Similar findings were
Asn-38 are shown in the site of PP processing in between Asn-38 and Val-39 (Fig. 1). This region corresponds to the SBT3-SBT3 complex (Figs. 3). With this mechanism and also with the exceptional stability of other complexes and facilitate PP degradation in a bimolecular complex. Once a free protease molecule is released, it can act on SBT3.

Five amino acids enriched at least 2-fold over natural abundance are shown for 4 result for the chymotrypsin library (149 unique peptides) is shown below. The amino acid sequence for the larger cleavage product matched best to the positions on either side of the cleavage site (P4 to P4'). Amino acids enriched at least 2-fold over natural abundance are shown for 4 positions on either side of the cleavage site (P4 to P4'). Amino acid distribution is expressed as percent difference (p = 0.003) to the natural abundance after normalization against the Swiss-Prot protein database for Arabidopsis thaliana, P, N, and T in positions P2, P1, and P3' matching the cleavage site at Asn-38 are shown in white letters for the trypsin (top) and chymotrypsin (bottom) libraries, respectively. Residues I, D, S, and K in positions P2, P1, P1', and P3' match the AspS2 cleavage site in SBT3PP and are depicted in black letters.

reported for bacterial and mammalian SBTs, where cleavage and degradation of the prodomain is slow and the rate-limiting step of enzyme maturation (26, 46). In subtilisin, PP degradation occurs in trans and requires the energetically unfavorable dissociation of the PP from the stable subtilisin-propeptide complex. Once a free protease molecule is released, it can act on other complexes and facilitate PP degradation in a bimolecular reaction (47). The slow activation of SBT3 (Fig. 9B) is consistent with this mechanism and also with the exceptional stability of the SBT3PP-SBT3 complex (Figs. 3B and 4B).

To identify the internal propeptide processing site, the two cleavage products (the bands marked in Fig. 9) were subjected to Edman degradation for N-terminal sequencing. The sequence for the larger cleavage product matched best to the loop region between strand β1 and helix α1, indicating cleavage between Asn-38 and Val-39 (Fig. 1). This region corresponds to the site of PP processing in Bacillus sp. subtilases (37, 48). Additional cleavage between Asp-54 and Ser-55 resulted in the generation of the smaller degradation product. As expected for an autocatalytic processing event, the amino acid sequence at these two sites agrees well with the substrate specificity of SBT3 that was determined by PICS (proteomic identification of cleavage sites; Ref. 49 and Fig. 9C). Pro-Asn immediately upstream of the first site as well as Ile-Asp and Ser-Xaa-Lys flanking the second are among the residues that are preferred by SBT3 in these positions, and they thus contribute to processing site recognition by the protease (Fig. 9C).

Discussion

We could show that the SBT3 propeptide acts as an intramolecular chaperone (IMC) that is required for folding of the protease in the plant secretory pathway and a prerequisite for secretion of mature SBT3 into the extracellular space. During protease maturation, the first autocatalytic cleavage of the PP results in the formation of a stoichiometric, autoinhibited complex of SBT3 and its PP. In this complex the PP acts as a strong inhibitor that renders SBT3 inactive until a second, pH-dependent cleavage event leads to the dissociation of the PP from the active site and results in the release of the active subtilase.

SBT3 shares these propeptide functions with prokaryotic and eukaryotic subtilases and combines features that were hitherto considered to be characteristic for either bacterial or for mammalian PPs. The ability of SBT3PP to acquire secondary structure independently of the catalytic domain is reminiscent of mammalian PPs (35, 37). Bacterial PPs, on the other hand, are generally disordered and depend on the interaction with the catalytic domain for the acquisition of secondary structure (38, 47, 50). There are exceptions, however, like the PP of the thermostable subtilisin homologue Aqualysin I from Thermus aquaticus, which develops significant secondary structure in absence of its catalytic domain (41).

With respect to primary structure, on the other hand, the PP of SBT3 is more similar to PPs of bacterial subtilisins. A large loop, which is located between strand β3 and helix α2 of the N2 motive in mammalian PPs, is not found in plants and bacteria. This is the region where secondary cleavage occurs in mammalian PPs, by which the subtilase is released from autoinhibition (29, 35, 48). In SBT3, on the other hand, the first of the two internal PP cleavage sites (Asn-38/Val-39) is located within the first β-α-β motive in a loop between strand β1 and helix α1. Conservation of this loop and the secondary cleavage site among bacterial and plant subtilases indicates similarities with respect to the mechanism of PP degradation and protease activation (35, 48).

Similarities with bacterial PPs extend to the mode of interaction between the PP and the catalytic domain, which was shown to differ for the immature and mature states of the protease, respectively. As an IMC, the PP of subtilisin interacts “top-on” and mediates folding of the intermediate, whereas it binds “side-on” when it acts as an inhibitor of the mature enzyme (21). Consequently, the IMC and inhibitor functions of bacterial PPs are not necessarily linked. The PP of Aqualysin I, for example, is a 10-fold more potent inhibitor of subtilisin E than its own PP but shows only half the chaperoning activity (41). Vice versa, the PP may also be a weak inhibitor while retaining high IMC.
Oligonucleotide primers used in this study

Sequences are given in the 5’ to 3’ orientation; F (forward) and R (reverse) refer to the direction of priming. Two cytosines were added at the 5’ end for increased efficiency of restriction. Restriction sites are underlined, and the used restriction enzyme is indicated in the primer name. All oligonucleotides were obtained from Eurofins Genomics GmbH (Ebersberg, Germany). Propeptides amplified for recombinant expression in E. coli lacked the N-terminal signal peptide (ΔSP) and included a tag of six histidine residues (His). Deletions (Δ) and insertions ([]) are indicated in the primer name and the name of the product as the range of amino acids given in one letter code with the position counted from the first Met.

| Primer sequence | Name | Product |
|-----------------|------|---------|
| 3-Kpn-ATG-F SBT3PP | | |
| 3P-Nde-HIS-F SBT3PP-HIS | | |
| 3-Bam-R SBT3PP | | |
| 1.1-Kpn-ATG-F SBT1.1PP | | |
| 1.1-Nde-HIS-F SBT1.1PP-HIS | | |
| 1.1-Bam-R SBT1.1PP | | |
| 1.2-Kpn-ATG-F SBT1.2PP | | |
| 1.4-Bam-R SBT1.4PP | | |
| 3-Bam-R SBT3PP | | |
| 1.3-Bam-R SBT1.3PP | | |
| 1.9.-Bam-R At1.9PP | | |
| 1.9.-Kpn-ATG-F At1.9PP | | |
| 1.2-Kpn-ATG-F SBT1.2PP | | |
| 1.4-Nde-HIS-F SBT1.4PP-HIS | | |
| 1.9.-Nde-HIS-F At1.9PP-HIS | | |
| 3P-Nde-HIS-F SBT3PP-HIS | | |
| 1.3-Nde-HIS-F SBT1.3PP-HIS | | |

Function of Propeptides in Plant Subtilases

TABLE 1

Oligonucleotide primers used in this study

Sequences are given in the 5’ to 3’ orientation; F (forward) and R (reverse) refer to the direction of priming. Two cytosines were added at the 5’ end for increased efficiency of restriction. Restriction sites are underlined, and the used restriction enzyme is indicated in the primer name. All oligonucleotides were obtained from Eurofins Genomics GmbH (Ebersberg, Germany). Propeptides amplified for recombinant expression in E. coli lacked the N-terminal signal peptide (ΔSP) and included a tag of six histidine residues (His). Deletions (Δ) and insertions ([]) are indicated in the primer name and the name of the product as the range of amino acids given in one letter code with the position counted from the first Met.

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| 1.1-Nde-HIS-F SBT1.1PP-HIS | | |
| 1.1-Bam-R SBT1.1PP | | |
| 1.2-Kpn-ATG-F SBT1.2PP | | |
| 1.4-Bam-R SBT1.4PP | | |
| 3-Bam-R SBT3PP | | |
| 1.3-Bam-R SBT1.3PP | | |
| 1.9.-Bam-R At1.9PP | | |
| 1.9.-Kpn-ATG-F At1.9PP | | |
| 1.2-Kpn-ATG-F SBT1.2PP | | |
| 1.4-Nde-HIS-F SBT1.4PP-HIS | | |
| 1.9.-Nde-HIS-F At1.9PP-HIS | | |
| 3P-Nde-HIS-F SBT3PP-HIS | | |
| 1.3-Nde-HIS-F SBT1.3PP-HIS | | |

activity (51). The inhibitor and IMC functions could also be separated in the PP of SBT3. A complete loss of IMC activity was observed when residues Ser-57 to Lys-70 were deleted from SBT3PP, whereas the activity as an inhibitor of mature SBT3 was only marginally impaired (Fig. 8), indicating that two different binding modes exist also in the interaction of SBT3PP with SBT3.

In addition to these shared features, SBT3PP also has unique properties. ThePPs from both prokaryotic and eukaryotic organisms have been described as quite promiscuous regarding their specificity toward the catalytic domain. Bacterial subtilisins and mammalian PCs alike are inhibited not only by their specificity toward the catalytic domain. Bacterial subtilisin, on the other hand, thermodynamic stability of the complex is reduced as compared with the free protease and only slightly higher than that of the folding intermediate (33, 52). Stabilizing a transition state late in the folding pathway, the PP catalyzes the reaction from the molten globule state to the autoinhibited complex as well as the back reaction (20, 24, 46). To prevent the back reaction, the PP needs to be cleaved, which kinetically locks the SBT in its active and stable conformation (52). Subtilisin maturation thus requires active subtilisin to assist in the maturation of other SBT-PP complexes by cleavage of the PPs in trans (46, 47). In the case of SBT3, increased stability of the complex as compared with both the mature protease and the folding intermediate renders the back reaction less favorable and traps the complex thermodynamically. Enhanced stability of the complex is likely to facilitate passage through the secretory pathway and prevent untimely activation until decreasing pH allows the controlled, pH-dependent cleavage of the PP and activation of SBT3.

Experimental Procedures

Cloning of Subtilase Propeptides—SBT PP s were cloned by PCR using gene-specific primers (Table 1) for SBT3PP (Sl01g087850), SBT1.1PP (Sl06g083720), SBT1.2PP (Sl08g007670), SBT1.3PP (Sl01g111400), SBT1.4PP (Sl01g087840), and AtSBT1.9PP (At5g67090). All constructs comprising the signal peptide, the propeptides, and flanking KpnI and BamHI restriction sites were cloned into pART7 (53) under the control of the cauliflower mosaic virus (CaMV) 35S promoter and terminator. The entire expression cassette was transferred into pART27 (53) for transient expression in plants. For expression in E. coli, a domain construct lacking the signal peptide were generated by PCR, digested with Ndel and BamHI, cloned with an N-terminal (His)6-tag into Novagen’s PET21a vector (Merck), and transformed into E. coli BL21 RIL (Agilent Technologies; Waldbronn, Germany).
Function of Propeptides in Plant Subtilases

Expression constructs were also generated for a SBT3 PP deletion mutant, lacking amino acids Ser-57 to Lys-70 (SBT3PPΔS-K), and SBT1.3PP with an insertion of the respective region (SBT1.3PP&K) using PCR-mediated deletion and insertion mutagenesis as described (54). Briefly, two fragments flanking the deletion/insertion-site were amplified in separate PCRs and then joined in a third PCR using the primers at the very 5’ and 3’ ends. For each mutant PP, two types of expression constructs were generated, one comprising the N-terminal signal peptide and KpnI/BamHI sites for cloning into pART7/27 constructs were generated, one comprising the N-terminal signal peptide and KpnI/BamHI sites for cloning into pART7/27 and expression in E. coli.

Expression and Purification of Recombinant Propeptides—E. coli cells were grown in LB medium with appropriate antibiotics at 220 rpm and 37 °C to an A600 of 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside during 16 h at 30 °C. Cells were harvested by centrifugation (5000 × g, 4 °C, 20 min) and resuspended in 50 mM sodium phosphate buffer, pH 7.5, supplemented with 300 mM NaCl and 5 mM β-mercaptoethanol. Cells were lysed by sonication (SONOPULS HB2070 with MS72 sonic needle, Bandelin Electronics; Berlin, Germany) at 4 °C for 3 min. The insoluble fraction was collected by centrifugation (12,000 × g, 4 °C, 30 min), and proteins were solubilized in buffer A (50 mM sodium phosphate buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole, 8 M urea) for 16 h at 4 °C on an end-over-end shaker. Cell debris was removed by centrifugation as above, and the cleared supernatant was subjected to affinity chromatography on nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany). The column was washed extensively in buffer A, recombinant proteins were eluted in 1.75 column volumes buffer A containing 400 mM imidazole, and their concentration was adjusted to 0.5 mg/ml. Renaturation of PPs was achieved by dialysis against 50 mM HEPES, pH 7.5, supplemented with 50 mM L-arginine hydrochloride and 50 mM L-glutamic acid.

Purification of SBT3 and Activity Assay—SBT3 was purified to apparent homogeneity from a tomato cell suspension culture as previously described (27). SBT3 activity was assayed in a total volume of 200 μl containing 20 μM aminobenzoyl-SKRDPKKQTD(NO2)Y (JPT Peptide Technologies; Berlin, Germany) and 90 nM concentrations of purified SBT3 in 50 mM HEPES, pH 7.5, 50 mM Arg, and 50 mM Lys, and subsequently against 20 mM sodium phosphate buffer, pH 7.5, in which the CD spectra were acquired. Spectra of 1.25 μM SBT3 or 15 μM SBT3PP were recorded in a 715 CD spectropolarimeter (Jasco; Hachioji, Japan) at 20 °C in the far UV (190 to 260 nm) with wavelength steps of 0.1 nm and a scan speed of 50 nm/min. The signal was averaged over four scans, and the buffer signal was subtracted.

Circular Dichroism (CD) Spectroscopy—SBT3 was denatured by dialysis against 50 mM HEPES, pH 7.5, containing 8 mM guanidine hydrochloride or by TCA precipitation. For renaturation, the protein was dialyzed first against 50 mM HEPES pH 7.5, 50 mM Arg, 50 mM Lys, and subsequently against 20 mM sodium phosphate buffer, pH 7.5, in which the CD spectra were acquired. Spectra of 1.25 μM SBT3 or 15 μM SBT3PP were recorded in a 715 CD spectropolarimeter (Jasco; Hachioji, Japan) at 20 °C in the far UV (190 to 260 nm) with wavelength steps of 0.1 nm and a scan speed of 50 nm/min. The signal was averaged over four scans, and the buffer signal was subtracted.

Fluorescence Spectroscopy—Fluorescence spectroscopy was performed at 20 °C in a Horiba FL-3 Fluorimeter (Jobin-Yvon; Kyoto, Japan). For tryptophan solvent accessibility tests, fluorescence of 1.25 μM SBT3 was recorded in 20 mM sodium phosphate buffer, pH 7.4, with excitation at 295 nm (slit 5 nm) and emission from 305 to 450 nm (slit 5 nm). For chemical titration experiments, GuHCl was titrated by stepwise addition to 1.25 μM SBT3 in 20 mM sodium phosphate buffer, pH 7.4, with an equilibration period of 3 min after each titration step using the same parameters for fluorescence spectroscopy. The background corrected fluorescence maxima from three independent experiments were plotted against the GuHCl concentration. Data were fitted to the Boltzmann equation using OriginPro 9.0 (OriginLabs; Northampton, MA).

Thermal Stability of SBT, SBT3PP, and the SBT3-SBT3PP Complex—Thermal stability of SBT3PP (20 μM) in 50 mM HEPES, pH 7.5, 50 mM Arg, and 50 mM Glu was analyzed in a Prometheus NT.48 instrument using the capillaries (10-μl sample volume) provided by the manufacturer (NanoTemper Technologies). Tryptophan fluorescence was measured at 350 and 330 nm, and the 350/330-nm ratio was plotted against the
temperature. Thermal denaturation was measured from 25 to 90 °C in the unfolding phase and from 90 to 25 °C for the refolding phase. Thermal unfolding of SBT3 and the SBT3-SBT3PP complex was recorded in a 715 CD-spectroptrometer (Jasco) at 220 nm in a thermal gradient with a step size of 0.1 °C and a slope of 1 °C per minute. The data were fitted using the Boltzmann equation to obtain the transition point (OriginPro 9.0, OriginLabs).

Identification of the Internal Propeptide Cleavage Site—Purified SBT3 was incubated with a 10-fold molar excess of its PP for 10 min at 4 °C. The SBT3-SBT3PP complex was then separated from excess PP by size exclusion chromatography (Superdex 200 HR 10/30) on an AKTA purifier 900 chromatography system (GE Healthcare). Fractions containing the complex were concentrated by ultrafiltration (Vivaspin, 10 kDa cutoff; Sartorius Stedim; Göttingen, Germany). For propeptide cleavage, ~15 μg of the SBT3-SBT3PP complex were incubated at 4 °C overnight in a three-component buffer system at pH 5.2 (56), separated by Tricine-SDS-PAGE (57), and blotted to a PVDF membrane (Trans-Blot Semi-Dry System; Bio-Rad). Proteins were visualized using 0.1% (w/v) Coomassie Brilliant Blue R250 in 40% ethanol and 10% acetic acid. Excised protein bands were subjected to N-terminal sequencing by Edman degradation on a Shimadzu PPSQ-33 sequencer (Creative Proteomics; Shirley, NY).

Transient Protein Expression in N. benthamiana—The pART27 constructs for transient expression of PPs were described above. Constructs for the expression of wild-type SBT3 and the S538A active-site mutant were described previously (27). An additional construct for a SBT3 mutant lacking the entire prodomain (ΔQ23-H112) was generated by PCR using two overlapping 5′ primers (Table 1) that comprised the open reading frame (ORF) for the signal peptide and 14 bp matching the 5′ end of the catalytic domain. The 3′ primer was located just downstream of an internal EcoRI site within the ORF of the catalytic domain. The PCR product was then used to replace the 5′ end of wild-type SBT3 to generate SBT3ΔPP.

Proteins were transiently expressed in N. benthamiana by co-infiltration of two Agrobacterium tumefaciens strains, one carrying the pART27 expression vector, the other carrying the p19 suppressor of silencing essentially as described (58). The bacterial suspension was infiltrated into the abaxial side of leaves from 6-week-old N. benthamiana plants. Leaves were harvested for protein extraction 4 days after inoculation.

Protein Extraction from N. benthamiana Leaves—For total protein extraction, leaf samples were ground in liquid nitrogen to a fine powder and thawed in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol containing 0.5% (v/v) Triton X-100, and a blend of protease inhibitors (SERVA Electrophoresis GmbH; Heidelberg, Germany). The extract was cleared by centrifugation (16,000 × g, 4 °C, 10 min) and kept at 4 °C until further analysis on the same day. The protocol for extraction of apoplastic wash fluids was modified from Rathmell and Sequeira (59). Freshly harvested leaves were rinsed 3 times in ice-cold water and vacuum-infiltrated with 50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl at 75 mbar. Apoplastic washes were recovered by centrifugation at 1500 × g at 4 °C for 7 min and used the same day for further analysis.

Analysis of SBT3 Substrate Specificity—SBT3 cleavage specificity was analyzed by PICS (49). For this assay total protein extracts from Arabidopsis seedlings were digested with proteomics-grade trypsin or chymotrypsin to generate libraries of several thousand peptides. These peptides were chemically modified to protect free sulfhydryl and amino groups and used as substrate for SBT3. Newly generated N termini were biotinylated, the peptides were isolated on streptavidin beads, and their sequence was determined by mass spectrometry (MS). This identifies the sequences on the carboxyl side (i.e. the prime side according to Schechter and Berger (60)) of all the bonds that were cleaved. With their sequence known, the sequences on the amino side (the non-prime side; Ref. 60) can be deduced and the cleavage sites reconstructed (49).

Libraries of tryptic and chymotryptic peptides were generated from protein extracts of 4-week-old Arabidopsis seedlings as described by Marino et al. (61) with minor modifications. 200 μg of the peptide libraries were digested with 2 μg of SBT3 for 16 h at pH 7.0 at 37 °C. N-terminally biotinylated peptides were purified over streptavidin-Sepharose (GE Healthcare) and analyzed via LC-MS/MS. A 75-μm analytical C18 column on an EASY-nLC HPLC system (Thermo Scientific) was used for separation with a linear acetonitrile gradient (4–64% in 135 min), and a Q-Exactive hybrid Orbitrap (Thermo Scientific) was used for mass analysis. MS/MS data were analyzed with MaxQuant (62). A web-based bioinformatics tool (WebPICS; Ref. 63) was used for reconstruction of cleavage sites.

Author Contributions—A. S. conceived and coordinated the study. M. M. and A. S. wrote the paper. M. M. designed, performed, and analyzed the experiments shown in Figs. 2, B and C, Fig. 4, and Figs. 6–9. M. W. cloned and purified the propeptides (Fig. 2A). S. L. performed and analyzed the experiments shown in Figs. 3, A and B, and 5. All authors reviewed the results and approved the final version of the manuscript.

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