Subtilase-mediated activation of CLEL peptides involves several processing events in consecutive compartments of the secretory pathway

Nils Stührwohldt[a], Stefan Scholl[b], Lisa Lang[a], Julia Katzenberger[a], Karin Schumacher[b], Andreas Schaller[a,2]

[a] Institute of Plant Physiology and Biotechnology, University of Hohenheim, 70593 Stuttgart, Germany.

[b] Department of Plant Developmental Biology, Centre for Organismal Studies, Heidelberg University, 69120 Heidelberg, Germany

[2]corresponding author: andreas.schaller@uni-hohenheim.de

Short title: Subcellular site of CLEL6 and CLEL9 maturation

ORCID identifiers:

AS: 0000-0001-6872-9576

NS: 0000-0003-4166-3786

Key words: Arabidopsis thaliana, post-translational modification, precursor processing, signaling peptide, subtilase.

Abstract

Small post-translationally modified peptides are emerging as a novel class of signaling molecules that are involved in many aspects of plant growth and development, and in the interaction of plants with their biotic environment. Despite considerable progress with respect to the receptor-mediated perception and signal transduction mechanisms, the maturation of these peptides from their larger precursors is still poorly understood. This question was addressed here for CLEL6 and CLEL9 (also known as GOLVEN 1 and 2) that are known to control gravitropic responses of the shoot and the root by modulation auxin distribution.

Several proteolytic processing events located in consecutive compartments of the secretory pathway were found to be required for the formation of bioactive CLEL peptides. Using an inhibitor-based approach for loss-of-function analysis, targeting protease function at the level of enzyme activity rather than gene expression, we show that the step-wise maturation of CLEL peptides is mediated by subtilases (SBTs). Following the cleavage of the signal peptide upon entry into the ER, the CLEL6 and 9 precursors are processed at two sites in their variable domain by SBT6.1. Cleavage by SBT6.1 in the cis-Golgi allows for continued passage of the partially processed (pre-activated) precursors through the secretory pathway, and is thus a prerequisite for subsequent post-translational modifications including tyrosine sulfation and proline hydroxylation within the Golgi, and proteolytic maturation after exit from the Golgi. The activation of CLEL6 and CLEL9 by SBTs in the trans-Golgi network or other post-Golgi compartments depends on the N-terminal aspartate of the mature peptides.

Introduction

Complementing the activity of the classical phytohormones, peptide hormones and growth factors are now recognized as an important class of signaling molecules for long-range signaling and for cell-to-cell communication over short distances, respectively (1, 2). In *Arabidopsis thaliana*, there are more than 1000 genes potentially encoding signaling peptides, apparently involved in all aspects of plant growth and development (3-5). There has been remarkable progress in recent years with respect to the characterization of peptide perception and signal transduction mechanisms (6, 7). The biogenesis of these signaling molecules, on the other hand, is still poorly understood. This is particularly true for the large group of signaling peptides that depend on a series of post-translational modifications (PTMs) for maturation and activation (2, 8).

Proteolytic processing is required for all post-translationally modified signaling peptides to release the peptide entity from its precursor. Additional PTMs may include tyrosine sulfation, proline hydroxylation, and arabinosylation of the hydroxyproline residue (2, 8). Tyrosine sulfation is performed by a single tyrosylprotein sulfotransferase (TPST) that is membrane-anchored in the cis-Golgi (9). TPST requires aspartate on the amino side of tyrosin for substrate recognition (9). Tyrosine sulfation is a critical maturation step, as sulfated peptides usually depend on this modification for full activity (2). Proline hydroxylation is catalyzed by membrane-anchored prolyl-4-hydroxylases (P4Hs) localized in ER and Golgi compartments. There are 13 P4Hs in Arabidopsis, some of which were shown to be required for the hydroxylation of extensin and possibly other hydroxyprolin (Hyp)-rich glycoproteins of the cell wall (10). Which of the P4Hs act on signaling peptides, and whether or not they differ in preference for proline in a certain sequence context is still unclear. Proline hydroxylation is a prerequisite for subsequent glycosylation. As the first in a series of glycosylation steps, *L*-arabinose is transferred to the 4-hydroxyl by Golgi-resident Hydroxyproline *O*-arabinosyltransferase (HPAT). HPAT is encoded by three genes in Arabidopsis that are at least partially functionally redundant (11, 12). To what extent differences in substrate specificity of HPATs may contribute to the selection of certain Hyp residues for glycosylation remains to be seen.

The identification of precursor processing proteases lags behind the other PTM enzymes. It was and is still hampered by the large number of possible candidates (907 peptidases are listed in the MEROPS database (release 12.0) for Arabidopsis (13)), by their frequently low expression levels, by functional redundancy, and by the lack of a conserved processing site. As compared to tyrosine

sulfation, proline hydroxylation and Hyp arabinosylation which occur at Asp-Tyr, Pro, and Hyp residues, respectively, and in contrast to animal systems, where peptide hormones are typically flanked by pairs of basic residues, there is no consensus motif that would mark the cleavage sites for processing of peptide precursors in plants (2, 13). We may thus expect that many different proteases with different specificities for cleavage site selection are required for the processing of the plentitude of precursors. Consistent with this notion, precursor processing enzymes have been identified in different classes of proteases, including a metacaspase and a papain-like enzyme among the cysteine peptidases (14, 15), a carboxypeptidase in the class of the zink-dependent metallo peptidases (16), and several subtilases (SBTs) among the serine peptidases (17-22). SBTs thus seem to play a more general role in peptide hormone maturation (23).

SBTs constitute a large family of mostly extracellular proteases including e.g. 56 members in Arabidopsis (24), 86 in tomato (25) and 97 in grapevine (26). Expansion of the SBT family in plants involved both whole genome and tandem gene duplications with differential neo- and sub-functionalization resulting in many taxon-specific clades (25, 27). AtSBT6.1 stands out in this diverse family for several reasons. First, unlike most other plant SBTs that are soluble proteins targeted to the cell wall (23), AtSBT6.1 is a membrane protein, anchored by a C-terminal membrane-spanning helix to the Golgi and possibly the plasma membrane (18, 28). Second, AtSBT6.1 is one of only two Arabidopsis SBTs that originated before the divergence of Metazoa and Viridiplantae, and is functionally conserved between animals and plants (23, 27). Like Site-1-Protease (S1P), its orthologue in humans, AtSBT6.1 (alias AtS1P) cleaves membrane-anchored bZIP transcription factors in the Golgi to facilitate the translocation of their cytoplasmic domain to the nucleus for the induction of ER stress-response genes (28, 29). Furthermore, the cleavage site preference of AtSBT6.1 for the amino acid motives R-(R/K)-X-L or R-(R/K)-L-X (18, 22) is almost identical with the substrate specificity reported for human S1P (R-X-(L/V/I)-X; (30)).

With its predominant Golgi localization (28, 31), AtSBT6.1 seems predestined to act in concert with TPST, P4Hs and HPATs in the biogenesis of post-translationally modified signaling peptides. Supporting this notion, potential AtSBT6.1/S1P cleavage sites can be found in many peptide precursors including members of the Rapid Alkalinization Factor (RALF), phytosulfokine (PSK), Clavata3/Embryo Surrounding Region (CLE) and CLE-Like (CLEL) families. The latter is also known as Golven (GLV) or Root Meristem Growth Factor (RGF) family and comprises 11 precursor-derived peptides of 13 to 18 amino acids carrying two additional post-translational

modifications, i.e. tyrosine sulfation and hydroxylation of the ultimate proline residue (32-34). We refer to them here as CLEL, because not all family members are involved in root gravitropism (causing the GLV phenotype), or regulating the activity of the root apical meristem (as the name RGF would suggest). However, processing by AtSBT6.1 has so far only been shown for RALF23 (21, 22), and in the particularly interesting case of CLEL6 (GLV1/RGF6)(18).

AtSBT6.1 was identified as a factor required for CLEL6 function in a screen for *sbt* mutants suppressing the CLEL6-overexpression phenotype (agravitropic root growth and increased hypocotyl elongation)(18). The protease was shown to cleave the CLEL6 precursor at two canonical AtSBT6.1/S1P cleavage sites (R-R-L-R, R-R-A-L), and both cleavage sites turned out to be relevant for CLEL6 function, the second one even essential. The data indicate that AtSBT6.1 activity is required for the formation of the bioactive CLEL6 peptide (18). Surprisingly however, AtSBT6.1 cleavage sites are located in the variable part of the CLEL6 and other peptide precursors, considerably upstream of the mature peptide sequence. AtSBT6.1 activity is thus not sufficient and additional unknown protease(s) are required for peptide maturation. Completely unresolved is the question when and where the processing of peptide precursors takes place, particularly in relation to the other PTMs. While the Golgi is an obvious possibility for processing by AtSBT6.1, the enzyme has also been reported at the cell surface (18) suggesting apoplastic processing of the fully modified precursor as an alternative possibility. This has implicitly been assumed for cell wall-localized SBTs. However, as secretory enzymes they are co-targeted with their potential peptide precursor substrates providing ample opportunity for processing *en route*, in any compartment of the secretory pathway. These are the questions that are addressed here for the CLEL6 and CLEL9 peptide precursors.

Results

SBT activity is required for the maturation of CLEL6 and CLEL9 peptides

In order to confirm the involvement of SBTs in the maturation of CLEL6 (GLV1/RGF6) and to address a possible involvement of SBTs in the processing of the CLEL9 (GLV2/RGF9) precursor, we used the inhibitor-based loss-of-function approach that was previously employed to demonstrate a role for redundant SBTs in the maturation of IDA (Inflorescence Deficient in Abscission) resulting in the shedding of Arabidopsis flower organs after pollination (19, 35, 36). The SBT-specific Extracellular Proteinase Inhibitors (EPIs) 1a and 10 from *Phytophthora infestans* were expressed in transgenic Arabidopsis plants under control of the *CLEL6* or *CLEL9* promoters (suppl. material, Fig. S1A). Inhibition of SBTs in tissues where the CLEL6 and 9 precursors activate the expression of EPIs is expected to phenocopy the *CLEL6/9* loss-of-function phenotype if SBT activity is required for precursor processing and peptide maturation.

Seedlings expressing EPI1a under the control of either the *CLEL6* or the *CLEL9* promoter were impaired in the gravitropic response of the hypocotyl (Fig. 1A,C). Likewise, hypocotyl gravitropism was inhibited also by the expression of EPI10 controlled by either one of the two *CLEL* promoters (suppl. material, Fig. S1B-D). The same phenotype had been observed when *CLEL6* or *9* were silenced in transgenic plants by artificial micro RNAs (33), suggesting that SBT activity is required for CLEL6 and 9 function. However, in contrast to *CLEL6* or *9*-silenced plants (33), the gravitropic response of roots was not affected in our EPI-expressing transgenics (suppl. material, Fig. S2). This observation is consistent with the fact that the *CLEL6* and *9* promoters are active in the hypocotyl where they drive the expression of EPI inhibitors in epidermis and cortex (33), but not in any part of the primary root (37).

When plants expressing the EPI1a inhibitor were supplied with synthetic CLEL6 or CLEL9 peptides, gravitropism of the hypocotyl was restored to wild-type level (Fig. 1B,D). Likewise, we observed an impaired gravitropic response in mutants defective in tyrosylprotein sulfotransferase (TPST), and the defect of the *tpst-1* mutant also was alleviated by application of the sulfated CLEL6 or CLEL9 peptides (Fig. 1E,F). The data indicate that the peptides act downstream of SBT (and TPST) activity, consistent with a role for SBTs (and TPST) in peptide maturation (Fig. 1B,D-F). The data are fully consistent with findings of Ghorbani et al. (18), who reported that the activity of

SBT6.1 is required for CLEL6 function, and they further indicate that SBTs are required also for the activation of CLEL9.

The CLEL6 precursor comprises two potential S1P (SBT6.1) cleavage sites, RRLR and RRAL (Fig. 2A), and the second site is necessary for CLEL6 function (18). However, cleavage by SBT6.1 is not sufficient for CLEL6 formation, since both sites are located considerably upstream of the mature peptide sequence. We thus refer to the cleavage by SBT6.1 as a necessary pre-processing step that precedes peptide activation (2). Additional protease(s) are needed to mark the N-terminus and release the fully processed CLEL6 peptide. Whether the final processing for peptide activation also is mediated by SBTs, is still unclear at this time. Also unclear are the subcellular sites of pre-processing and peptide activation, and the sequence of post-translational modification events. These questions were addressed in the following.

Pre-processing by SBT6.1 in an early Golgi compartment is required for secretion

SBT6.1 is known to be active in the Golgi, where it is required for the processing and activation of membrane-anchored transcription factors and of PMEs (28, 29, 38, 39), and it was reported also in the cell wall, where it was detected in complex with the Serpin1 inhibitor (18). To address the question whether pre-processing by SBT6.1 occurs within the secretory pathway or extracellularly, we transiently expressed the CLEL6 precursor fused to sfGFP in *N. benthamiana*. The sfGFP tag was linked to the N-terminus of the precursor, just downstream of the signal peptide (construct named Sec in Fig. 2B). GFP fluorescence was detected only in the apoplast (Fig. 2D). On an anti-GFP immunoblot a single band was detected corresponding in size to GFP with part of the precursor up to the first SBT6.1 cleavage site (Fig. 2E). Processing at this site is thus efficient when the precursor is allowed to pass through the secretory pathway.

When the precursor was equipped with a C-terminal KDEL-motif for ER retention (construct named KDEL in Fig. 2B), processing was incomplete (Fig. 2E). In addition to the apoplast, the fluorescence signal was now detected also in the ER and Golgi, as indicated by co-expression of ER (Vma12-mRFP) or Golgi (ManI-mCherry and ST-mCherry for early and late Golgi, respectively) markers (Fig. 2G). The signal in the ER and early Golgi compartments results from the unprocessed precursor, while the presence of extracellular GFP indicates that some of the precursor was processed to separate GFP from the ER retention signal. Partial processing also is

apparent on the western blot, where two additional bands were observed (Fig. 2E), which we interpret as the precursor processed at the second SBT6.1 site, and the unprocessed precursor, respectively.

ER retention by the KDEL-motif is mediated by Golgi-resident K/HDEL-receptors, which effect retrograde transport of soluble ER proteins from the Golgi back to the ER (40-42). Cleavage by SBT6.1 may thus occur either in the ER or in the Golgi. However, processing was abolished when anterograde ER-to-Golgi vesicle transport was inhibited by addition of brefeldin A (43) (Fig. 2C). These observations indicate that exit from the ER is required for cleavage by SBT6.1, and we conclude that SBT6.1 acts in the Golgi, likely in an early Golgi compartment. This conclusion was confirmed by fusing the CLEL6 precursor to the N-terminal membrane anchor of ß-1,2-xylosyltransferase (XylT), which is sufficient to target reporter proteins to the *medial* Golgi (44)(Fig. 2H). The same three cleavage products were observed as for the KDEL-tagged precursor at somewhat different ratios (Fig. 2E) indicating that cleavage by SBT6.1 occurs before the precursor reaches the *trans* Golgi network (TGN).

To assess the relevance of cleavage by SBT6.1 for processing and secretion, we masked both cleavage sites (Fig. 2A) by alanine substitutions and analyzed the effect on the processing pattern of the transiently expressed precursor (compare constructs Sec and Sec^m in Fig. 2F). The central band corresponding to the second cleavage site was lost for the Ala-substituted precursor confirming that SBT6.1 is responsible and necessary for this cleavage event. This may not be the case for the first cleavage event, as the corresponding band was still observed for the Ala-substituted precursor, suggesting that another protease may jump in when cleavage by SBT6.1 is prevented. Interestingly, comparing Figures 2D,I, we observed that secretion of the Ala-substituted precursor is reduced compared to the wild-type. For the Ala-substituted precursor, the GFP fluorescence signal was observed in both ER and Golgi in addition to the apoplastic space suggesting that cleavage by SBT6.1 may be a prerequisite for continued passage along the secretory pathway and, hence, for additional post-translational modifications in post-Golgi compartments. This observation may explain why pre-processing by SBT6.1 is required for CLEL6 function *in vivo* (18), despite the fact that this cleavage event does not produce the mature peptide.

The cleavage for final activation occurs in a post-Golgi compartment by an aspartate-dependent SBT

After pre-processing of CLEL6 by SBT6.1 in the Golgi, additional processing at the N-terminus is required for maturation and activation. To localize this processing event subcellularly, we used an N-terminally sfGFP-tagged deletion construct of the CLEL6 precursor (Δ-Sec) lacking both SBT6.1 processing sites (Fig. 3A). Again, we analyzed a secreted version (Δ-Sec), one that was equipped with a C-terminal KDEL-motif for ER retention (Δ-KDEL) and one that was anchored to the Golgi membrane (Δ-XylT, Fig. 3B). On an anti-GFP immunoblot a single band was detected for Δ-Sec corresponding in size to the precursor processed at the N-terminal maturation site (Fig. 3C). Interestingly, for Δ-Sec some of the GFP signal was observed in the cell wall, in addition to the ER and a weak signal in the late Golgi (ST-mCherry marker in Fig. 3D). Secretion of this construct, like that of Sec^m (Fig. 2I), is thus reduced compared to wild-type Sec (Fig. 2D), suggesting once again that the propeptide and propeptide cleavage by SBT6.1 may be needed for efficient passage through the secretory pathway.

In contrast to Δ-Sec, the unprocessed precursors were observed for both Δ-KDEL and Δ-XylT as single larger bands on the immunoblot (Fig. 3C). The GFP signal for the C-terminally KDEL-tagged deletion (Δ) construct was found exclusively in the ER (Fig. 3E). Retention of the N-terminal GFP tag in the ER confirmed that processing did not occur, indicating that the maturation step is located further downstream in the secretory pathway. For Δ-XylT the apparently unprocessed precursor (Fig. 3C) co-localized exclusively with Golgi markers (Fig. 3F), suggesting a post-Golgi compartment or, at the latest, the apoplastic space as the site for CLEL6 maturation. Maturation late in the secretory pathway was also observed for CLEL9. Similar to CLEL6, the Δ-KDEL and Δ-XylT constructs for CLEL9 were not processed and were retained in the ER and Golgi, respectively (suppl. material, Fig. S3). For the Δ-Sec construct that is allowed to proceed beyond the Golgi, on the other hand, the smaller, processed product was generated (suppl. material, Fig. S3B). Our data thus indicate that both CLEL6 and CLEL9 mature after exit from the Golgi, in the TGN, in secretory vesicles, or in the apoplastic space.

All CLEL peptide precursors including both CLEL6 and 9 share a conserved aspartate upstream of the sulfated tyrosine (Fig. 3A). To test whether this aspartate is necessary for peptide processing and/or activity, we generated site-directed D-to-A mutants of both CLEL6 (D71A) and CLEL9 (D66A) and compared processing to the corresponding wild-type versions (Fig. 4A,B). As

compared to the fully processed Δ-Sec constructs of CLEL6 and CLEL9, the larger unprocessed form was observed for the Δ-Sec D71A and Δ-Sec D66A mutants, indicating that the aspartate is indeed required for processing (Fig. 4A,B). When fused to the XylT Golgi anchor, the processing-resistant D71A and D66A mutants exhibited the same apparent molecular weight as the wild-type Δ-XylT constructs (Fig. 4A,B), thus confirming that the band produced from the wild-type Δ-XylT construct of CLEL6 corresponds to the full-length precursor, despite its faster migration as compared to the unprocessed Δ-KDEL band (Fig. 3C).

A bioassay was then used to assess whether the aspartate and aspartate-dependent processing are required for bioactivity. CLEL6 and CLEL9 peptides induce waving of Arabidopsis roots (33), with half-maximal activities of about 3 and 100 nM, respectively (suppl. material, Fig. S4). The full-length Sec CLEL6 and CLEL9 constructs were transiently expressed in *N. benthamiana* and any peptides produced from these precursors were extracted in apoplastic washes. When Arabidopsis seedlings were treated with equal amounts of these cell wall extracts, root waving (quantified as changes in growth direction of the primary root) was induced indicating the formation of bioactive CLEL6 and CLEL9 peptides (Fig. 4C-E). Bioactivity was significantly reduced for the alanine-substituted precursors (Fig. 4C,E) confirming the importance of the aspartate for peptide activation.

The protease(s) required in addition to SBT6.1 for the final maturation step and activation of CLEL6 and CLEL9 is still unknown. To test a potential involvement of SBTs, as it was suggested by the impaired gravitropic response of the hypocotyl in seedlings expressing EPI1a or EPI10 under the control of either the *CLEL6* or the *CLEL9* promoter (Fig. 1A,C*;* suppl. material, Fig. S1B-D), the flag-tagged EPI10 inhibitor was co-expressed with the Δ-Sec CLEL6 and CLEL9 constructs in *N. benthamiana* (suppl. material, Fig. S5). As compared to the single, fully processed band that was observed again for Δ-Sec CLEL6 and Δ-Sec CLEL9, the co-expression of EPI10 reduced the efficiency of processing resulting in additional bands corresponding to the unprocessed CLEL6 and CLEL9 precursors (suppl. material, Fig. S5). The data indicate that on top of SBT6.1, an additional SBT(s) is required for the maturation of CLEL peptides. The identity of this SBT(s), of which there are 56 in Arabidopsis, remains to be addressed in future studies.

Discussion

Using an inhibitor-based approach targeting SBT function at the level of enzyme activity rather than gene expression, we confirmed that SBTs are required for the gravitropic response of etiolated Arabidopsis seedlings (Fig. 1A,C; suppl. material, Fig. S1C-D). The loss-of-function phenotype of EPI1a and EPI10-expressing transgenic plants was complemented by application of the mature CLEL6 and CLEL9 peptides (Fig. 1B,D), indicating that SBT activity is required upstream of the peptides, consistent with a role in peptide formation. SBT6.1 and SBT6.2 were previously shown to be necessary for CLEL6 function in a screen for *sbt* mutants suppressing the overexpression phenotype of the CLEL6 precursor (18). Consistently, two SBT6.1 cleavage sites were identified in the precursor, and the second site was found indispensable for CLEL6 activity (18). While the distantly related SBT6.2 also seemed to be necessary for CLEL6 function in this system (18), its cytosolic/nuclear localization, and its activity as tripeptidyl peptidase in general protein turnover (45, 46) are difficult to reconcile with any direct contribution to peptide maturation, which would require co-localization of enzyme and substrate within the secretory pathway. Further, due to the cytosolic/nuclear localization of SBT6.2, it cannot be targeted by the secreted EPI inhibitors and, therefore, the phenotype of EPI overexpressors cannot be explained by the inhibition of SBT6.2 activity. We thus exclude a direct involvement of SBT6.2 in CLEL6 and CLEL9 processing.

Addressing the sequence and subcellular sites of maturation events we show here that several consecutive processing steps are required for the biogenesis of CLEL peptides in Arabidopsis. Cleavage of CLEL precursors by SBT6.1 constitutes the first obligatory processing step in peptide maturation (not considering the co-translational cleavage of the signal peptide). However, the subcellular site of SBT6.1-mediated processing remained unresolved, as SBT6.1 was reported to be dually localized in the cis-Golgi and at the plasma membrane. We show here that SBT6.1 cleaves CLEL precursors soon after exit from the ER in an early Golgi compartment (Fig. 2), indicating that the reported plasma-membrane localization is irrelevant for the maturation of CLEL6 and 9.

Site-directed mutagenesis of SBT6.1 cleavage sites impaired secretion of the CLEL precursors, as some of the signal got stuck in the ER and the Golgi (Fig. 2I,F). The variable pro-region of the precursor including the SBT6.1 cleavage sites is thus important for secretion. Similarly in the animal field, neurotrophins are synthesized as larger pro-proteins that need proteolytic processing to yield mature and biologically active neurotrophic factors, which play

important roles in the development, maintenance and regeneration of the nervous system (47). For brain-derived and glial cell-line derived neurotrophic factors (BDNF and GDNF, respectively) the cleavable prodomain was found to be required for post-Golgi trafficking. Sorting of BDNF and GDNF to secretory granules depends on sorting receptors of the Vps10p (vacuolar protein-sorting 10 protein) family, sortilin and sorLA, respectively (48, 49). Sortilin also facilitates prodomain-dependent export of hydrophobic conotoxins from the ER, by allowing them to escape ER quality control mechanisms (50). Likewise, the prodomain of CLEL peptides may interact with unidentified sorting receptors to facilitate secretion or, alternatively, cleavage of the prodomain by SBT6.1 may provide a point of quality control, before the now partially processed precursor is allowed to leave the Golgi for final activation.

The requirement of SBT6.1-mediated processing for secretion provides an explanation for the perplexing finding that SBT6.1 cleavage sites and, by inference, cleavage by SBT6.1 are required for CLEL6 activity (18), despite the fact that cleavage at these sites does not produce the active peptide. We show here that this second obligatory cleavage event marking the N-termini of the mature CLEL6 and 9 peptides takes place in a post-Golgi compartment, i.e. in the TGN, in secretory vesicles, or ultimately in the apoplastic space. Our data suggest that cleavage by SBT6.1 allows for continued passage of partially processed (pre-activated) precursors through the secretory pathway, and is thus a prerequisite for subsequent post-translational modifications in the Golgi (sulfation, proline hydroxylation) and post-Golgi compartments (proteolytic maturation) (Fig. 5).

The second obligatory processing event and formation of the bioactive peptide hinged on the N-terminal aspartate residue (Fig. 4A-E). This processing step was inhibited by the SBT-specific inhibitor EPI10 (51)(suppl. material, Fig. S5), indicating that cleavage at the N-terminus of CLEL6 and 9 peptides also is mediated by SBT(s), particularly by Asp-dependent SBT(s). Unlike SBT6.1 and 6.2, all other members of the plant SBT family are secretory enzymes (23) that co-migrate with potential pro-peptide substrates through the secretory pathway towards the cell wall as their final destination, thus providing ample opportunity for interaction *en route*. Nonetheless, cleavage does not occur before the partially processed precursor leaves the Golgi. We see two possible explanations for this apparent discrepancy. First, the precursor may not be fit for cleavage, or second, the protease may not be active before exit from the Golgi.

The first scenario implies that post-translational modifications in earlier compartments are mandatory for subsequent SBT-mediated cleavage. Interestingly, tyrosin sulfation by TPST is

known to depend on an adjacent aspartate residue (9, 52). Therefore, if the apparent aspartate-dependency of the N-terminal maturation step is only indirect, and the SBT responsible for this processing event rather needs sulfotyrosin for cleavage site recognition, post-translational modification by Golgi-resident TPST would be a prerequisite for SBT-mediated cleavage. This scenario is supported by the impaired gravitropic response of the *tpst-1* mutant that was rescued by addition of sulfated CLEL6 and 9 peptides indicating that tyrosine sulfation is indeed required for peptide biogenesis (Fig. 1E,F). However, we cannot distinguish at this time whether tyrosine sulfation is required for cleavage site recognition by the processing SBT, or else, for ligand binding by the cognate receptors. This question will be resolved once the elusive SBT and CLEL6/9 receptor(s) are identified and characterized.

Alternatively, and more directly supported by the available data, it may be the control of SBT activity rather than co-localization in the secretory pathway that determines the subcellular site of proteolytic pro-peptide activation. In general, SBT activity is controlled by the prodomain that acts as an intramolecular chaperone for folding, and as an inhibitor of the mature enzyme (53). SBT zymogens remain inactive until the prodomain is cleaved off auto-catalytically and subsequently released (53-55). Prodomain-mediated inhibition and latency of the zymogen are pH-dependent, and broken in a compartment-specific manner as the pH drops along the secretory pathway. In the well-studied case of furin, for example, the prodomain is released in a second autocatalytic cleavage event, which does not occur before the zymogen reaches the acidic environment of the TGN (55). Similarly, SBT3 from tomato also requires the acidic pH of post-Golgi compartments for prodomain cleavage and activation (53). These findings may explain why the second obligatory cleavage event does not occur before peptide precursors exit the Golgi, and they support the TGN as the site for CLEL 6 and 9 maturation. However, there is precedence for SBTs that remain inactive until they reach the cell wall. In case of SBT1 from tomato, an N-terminal inhibitory peptide of 21 amino acids keeps the enzyme inactive even after removal of the prodomain. Cleavage of this auto-inhibitory peptide requires a further drop in pH and, therefore, it occurs only when the zymogen is secreted into the cell wall (56). We thus cannot exclude the apoplast as the site for final maturation and activation of CLEL peptides. Identification and characterization of the SBT responsible will ultimately resolve this question.

Methods

Plant material and growth conditions

For growth experiments in axenic culture, Arabidopsis seeds were surface-sterilized in 70 % ethanol for 15 minutes, washed in 100 % ethanol and laid out in rows on square plates containing 0.5 x MS (Murashige-Skoog) medium (35), 1 % sucrose and 0.38 % gelrite. Seeds were stratified for two days at 4 °C and grown for 5 days in the dark. For quantitative analysis of gravitropic responses, 5-day old vertically grown seedlings were rotated for 90° in the dark and grown for further two days. The bending angle was measured using ImageJ (http://rsbweb.nih.gov/ij/). All experiments were carried out at least three times with similar results. If indicated, media were supplemented with synthetic CLEL6 (DY(SO_3H)PQPHRKPPIHNE) or CLEL9 (DMDY(SO_3H)NSANKKRPIHNR) peptides (PepMic; Suzhou, China) at the indicated concentrations.

Generation of expression constructs

The PCR primers used for amplification of CLEL6/9 precursors and tags are listed in suppl. material, Table S1. As a general strategy, PCR products with flanking restriction sites were first cloned into pCR2.1-Topo (Life Technologies; Carlsbad, California, USA) and verified by sequencing (Macrogen; Amsterdam, The Netherlands). Using the flanking restriction sites (suppl. material, Table S1), the inserts were mobilized from pCR2.1-Topo and cloned into pART7 (57) between the cauliflower mosaic virus (CaMV) 35S promoter and terminator. The entire expression cassette was then transferred into the *NotI* site of pART7 (57) for transient expression in plants. Strains C58C1 or GV3101 were used for Agrobacteria-mediated expression. More specifically, for the generation of constructs with sfGFP (58) inserted between the N-terminal signal peptide or the XylT35 membrane anchor and the CLEL propeptide sequences, overlapping PCR was used to fuse the ORFs of the CLEL6 signal peptide or the first 35 amino acids of 1,2-xylosyltransferase (44) to the 5'-end of sfGFP. For the generation of CLEL6 and CLEL9 constructs in C-terminal fusion to sfGFP, the propeptide ORFs were cloned into the *Eco*RI site of pART7. Orientation was tested by PCR and sequencing. The ORF of sfGFP with C-terminal hexa-His tag was cut out from pMS119EH-sfGFP (59) with *Bam*HI and *Hin*dIII, and subcloned into the *Bam*HI and *Xba*I sites of pART7, in translational fusion with the propeptide ORFs (*Hin*dIII and XbaI sites were blunted). For ER retention, the ORF of sfGFP was extended by PCR to include the C-terminal KDEL

sequence, and the PCR product replaced the sfGFP-hexa-His ORF in pART7. CLEL6 and CLEL9 were N-terminally coupled to sfGFP by *Eco*RI and *Bam*HI. For constructs expressing EPI1a and EPI10 under the control of the CLEL6 or CLEL9 promoters, EPI1a and EPI10 constructs described by Schardon et al. (19) were used as a starting point. EPI1a, modified with a flag tag insertion between the signal peptide and the inhibitor, and EPI10-flag ORFs were amplified by PCR to include *Eco*RI and *Xho*I restriction sites and ligated into the corresponding restriction sites of pGreen0029, upstream of the *nptII* terminator sequence. *CLEL6* and *CLEL9* promoters (33) were PCR-amplified with terminal *Not*I and *Eco*RI restriction sites, and ligated into the corresponding sites of pGreen229, upstream of EPI1a and EPI10, respectively. Plasmids were transformed into GV3101 containing the pSOUP helper plasmid and transformed into *Arabidopsis thaliana* Col 0 by floral dip (60). Transgenic lines were selected on glufosinate and homozygous lines in the T3 or T4 generation were used in further experiments.

The expression construct for the VMA12-mRFP ER marker has been described previously (61). The expression constructs $VHP1_{Pro}$*:ManI-mCherry* and $VHP1_{Pro}$*:ST-mCherry* for Golgi markers were generated using the GreenGate cloning system (62). GreenGate modules used are listed in suppl. material, Table S2. To generate new entry modules, fragments were PCR amplified from pre-existing plasmids, *Arabidopsis thaliana* Col-0 genomic DNA or cDNA with "Phusion High-Fidelity DNA-Polymerase" (Thermo Scientific; Waltham, MA). After purification, PCR-products were digested with *Eco*31I-HF (Thermo Scientific) to open module specific overhangs. Fragments were then ligated in *Eco*31I-opened and purified entry vectors. Presence and sequence of inserts were verified via restriction digest and sequencing.

Transient expression in *N. benthamiana* and protein extraction

A. tumefaciens strains C58C1 and GV3101 were used for transient expression in *N. benthamiana*. Bacteria were grown on plates containing appropriate antibiotics (Rifampicin, tetracycline and spectinomycin for C58C1 and gentamycin and spectinomycin for GV3101) at 28 °C and were washed off the plates in 10 mM MES, pH 5.6 containing 10 mM $MgCl_2$. A blunt syringe was used to infiltrate the bacterial suspension supplemented with 150 µM acetosyringone into the leaves. For total protein extraction, leaves were harvested two to three days after infiltration into liquid nitrogen and ground to a fine powder. The powder was thawed in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 10 mM β-mercaptoethanol containing 0.5% Triton X-100 and proteinase inhibitor

mix P (#39103, SERVA Electrophoresis GmbH; Heidelberg, Germany). The extracts were centrifuged (16.000 g, 4 °C, 10 min) and the supernatant was kept at 4 °C until usage at the same day, or frozen at -20°C.

Immunodetection

For western blots, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using standard procedures. Polyclonal anti-GFP antibodies (1:10000; A-11122, Thermo Fisher Scientific) or anti-Flag antibodies (1:5000; Sigma-Aldrich; Taufkirchen, Germany) directly coupled to horseradish peroxidase were used for immunodetection, followed by enhanced chemiluminescence detection with an Odyssey Fc imager (Li-COR Biotechnology; Homburg, Germany).

Fluorescence Microscopy

Agro-infiltrated leaves of *N. benthamiana* were observed with a TCS SP5 II inverted Confocal Laser Scanning Microscope (Leica Microsystems; Wetzlar Germany) using a HCX PL APO lambda blue 63.0 x 1.20 water immersion objective (Leica Microsystems). sfGFP was excited with the 488 nm line of the VIS-Argon laser; for mRFP/mCherry the 561 nm line generated by a VIS-DPSS 561 laser was used. Emission was detected at 500-550 nm for sfGFP and 610-670 nm for mRFP/mCherry with HyD hybrid detectors (Leica Microsystems) in standard operation-mode. Autofluorescence was detected between 700-800 nm with identical laser settings as used for sfGFP-mRFP/mCherry image recording. Images were adjusted in brightness and processed using "Mean" Filter with a pixel radius of 0.1 with ImageJ software version 1.51s (National Institute of Health).

Supplemental Information

Supplemental Figures S1 to S5

Supplemental Tables S1 to S2

Author Contributions

NS, LL, JK and SS performed experiments, NS and AS designed the research strategy, KS and AS supervised the research and NS and AS wrote the paper.

Acknowledgements

Authors thank Lhana Stein, Bianca Bukowski and Jutta Babo for excellent technical support. We thank Dr. Rainer Waadt for contributing the pGGA-VHP1 entry module.

References

1. Oh E, Seo PJ, & Kim J (2018) Signaling peptides and receptors coordinating plant root development. *Trends Plant Sci* 23:337-351.
2. Stührwohldt N & Schaller A (2018) Regulation of plant peptide hormones and growth factors by post-translational modification. *Plant Biol* early online publcation:doi:10.1111/plb.12881.
3. Lease KA & Walker JC (2006) The Arabidopsis unannotated secreted peptide database, a resource for plant peptidomics. *Plant Physiol* 142:831-838.
4. Ghorbani S, *et al.* (2015) Expanding the repertoire of secretory peptides controlling root development with comparative genome analysis and functional assays. *J Exp Bot* 66:5257-5269.
5. Tavormina P, De Coninck B, Nikonorova N, De Smet I, & Cammue BPA (2015) The plant peptidome: An expanding repertoire of structural features and biological functions. *Plant Cell* 27:2095-2118.
6. Song W, Han Z, Wang J, Lin G, & Chai J (2017) Structural insights into ligand recognition and activation of plant receptor kinases. *Curr Opin Struct Biol* 43:18-27.
7. He Y, Zhou J, Shan L, & Meng X (2018) Plant cell surface receptor-mediated signaling - A common theme amid diversity. *J Cell Sci* 131: jcs209353.
8. Matsubayashi Y (2014) Posttranslationally modified small-peptide signals in plants. *Ann Rev Plant Biol* 65:385-413.
9. Komori R, Amano Y, Ogawa-Ohnishi M, & Matsubayashi Y (2009) Identification of tyrosylprotein sulfotransferase in Arabidopsis. *Proc Natl Acad Sci USA* 106:15067-15072.
10. Velasquez Silvia M, *et al.* (2015) Complex regulation of prolyl-4-hydroxylases impacts root hair expansion. *Mol Plant* 8:734-746.
11. Ogawa-Ohnishi M, Matsushita W, & Matsubayashi Y (2013) Identification of three hydroxyproline *O*-arabinosyltransferases in *Arabidopsis thaliana*. *Nature Chem Biol* 9:726-730.
12. MacAlister CA, Ortiz-Ramírez C, Becker JD, Feijó JA, & Lippman ZB (2016) Hydroxyproline *O*-arabinosyltransferase mutants oppositely alter tip growth in *Arabidopsis thaliana* and *Physcomitrella patens*. *Plant J* 85:193-208.
13. Rawlings ND, Barrett AJ, & Finn R (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucl Acids Res* 44:D343-350.

14. Wrzaczek M, *et al.* (2015) GRIM REAPER peptide binds to receptor kinase PRK5 to trigger cell death in Arabidopsis. *EMBO J* 34:55-66.
15. Ziemann S, *et al.* (2018) An apoplastic peptide activates salicylic acid signalling in maize. *Nature Plants* 10.1038/s41477-018-0116-y.
16. Casamitjana-Martínez E, *et al.* (2003) Root-specific *CLE19* overexpression and the *sol1/2* suppressors implicate a CLV-like pathway in the control of *Arabidopsis* root meristem maintenance. *Current Biol* 13:1435-1441.
17. Engineer CB, *et al.* (2014) Carbonic anhydrases, EPF2 and a novel protease mediate CO_2 control of stomatal development. *Nature* 513:246-250.
18. Ghorbani S, *et al.* (2016) The SBT6.1 subtilase processes the GOLVEN1 peptide controlling cell elongation. *J Exp Bot* 67:4877-4887.
19. Schardon K, *et al.* (2016) Precursor processing for plant peptide hormone maturation by subtilisin-like serine proteinases. *Science* 354:1594-1597.
20. Beloshistov RE, *et al.* (2018) Phytaspase-mediated precursor processing and maturation of the wound hormone systemin. *New Phytol* doi:10.1111/nph.14568:1167–1178.
21. Stegmann M, *et al.* (2017) The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling. *Science* 355:287-289.
22. Srivastava R, Liu J-X, Guo H, Yin Y, & Howell SH (2009) Regulation and processing of a plant peptide hormone, AtRALF23, in Arabidopsis. *Plant J* 59:930-939.
23. Schaller A, *et al.* (2018) From structure to function – a family portrait of plant subtilases. *New Phytol* 218:901-915.
24. Rautengarten C, *et al.* (2005) Inferring hypotheses on functional relationships of genes: Analysis of the *Arabidopsis thaliana* subtilase gene family. *PLoS Comput Biol* 1:e40.
25. Reichardt S, *et al.* (2018) The tomato subtilase family includes several cell death-related proteinases with caspase specificity. *Sci Rep* 8:10531.
26. Figueiredo J, *et al.* (2016) Revisiting Vitis vinifera Subtilase Gene Family: A Possible Role in Grapevine Resistance against Plasmopara viticola. *Front Plant Sci* 7:1783.
27. Taylor A & Qiu Y-L (2017) Evolutionary history of subtilases in land plants and their involvement in symbiotic interactions. *Mol Plant-Microbe Int* 30:489-501.
28. Liu J-X, Srivastava R, Che P, & Howell SH (2007) Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J* 51:897-909.

29. Liu J-X, Srivastava R, Che P, & Howell SH (2007) An endoplasmic reticulum stress response in *Arabidopsis* is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. *Plant Cell* 19:4111-4119.
30. Seidah NG (2013) Chapter 721 - Site-1 Protease. *Handbook of Proteolytic Enzymes (Third Edition)*, eds Rawlings ND & Salvesen G (Academic Press), pp 3265-3270.
31. Parsons HT, *et al.* (2012) Isolation and proteomic characterization of the Arabidopsis Golgi defines functional and novel components involved in plant cell wall biosynthesis. *Plant Physiol* 159:12-26.
32. Matsuzaki Y, Ogawa-Ohnishi M, Mori A, & Matsubayashi Y (2010) Secreted peptide signals required for maintenance of root stem cell niche in *Arabidopsis*. *Science* 329:1065-1067.
33. Whitford R, *et al.* (2012) GOLVEN secretory peptides regulate auxin carrier turnover during plant gravitropic responses. *Dev Cell* 22:678-685.
34. Meng L, Buchanan BB, Feldman LJ, & Luan S (2012) CLE-like (CLEL) peptides control the pattern of root growth and lateral root development in *Arabidopsis*. *Proc Natl Acad Sci U S A* 109:1760-1765.
35. Stührwohldt N, Schardon K, Stintzi A, & Schaller A (2017) A toolbox for the analysis of peptide signal biogenesis. *Mol Plant* 10:1023-1025.
36. Stührwohldt N, Hohl M, Schardon K, Stintzi A, & Schaller A (2018) Post-translational maturation of IDA, a peptide signal controlling floral organ abscission in Arabidopsis. *Commun Integ Biol* 11:e1395119.
37. Fernandez A, *et al.* (2013) Transcriptional and functional classification of the GOLVEN/ROOT GROWTH FACTOR/CLE-Like signaling peptides reveals their role in lateral root and hair formation. *Plant Physiol* 161:954-970.
38. Senechal F, *et al.* (2014) Arabidopsis PECTIN METHYLESTERASE17 is co-expressed with and processed by SBT3.5, a subtilisin-like serine protease. *Ann Bot* 114:1161-1175.
39. Wolf S, Rausch T, & Greiner S (2009) The N-terminal pro region mediates retention of unprocessed type-I PME in the Golgi apparatus. *Plant J* 58:361-375.
40. Phillipson BA, *et al.* (2001) Secretory bulk flow of soluble proteins Is efficient and COPII dependent. *Plant Cell* 13:2005-2020.
41. Pelham HR (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J* 7:913-918.

42. Silva-Alvim FAL, *et al.* (2018) Predominant Golgi residency of the plant K/HDEL receptor is essential for its function in mediating ER retention. *Plant Cell* 30:2174-2196.
43. Nebenfuhr A, Ritzenthaler C, & Robinson DG (2002) Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol* 130:1102-1108.
44. Pagny S, *et al.* (2003) Structural requirements for Arabidopsis beta1,2-xylosyltransferase activity and targeting to the Golgi. *Plant J* 33:189-203.
45. Book AJ, Yang PZ, Scalf M, Smith LM, & Vierstra RD (2005) Tripeptidyl peptidase II. An oligomeric protease complex from *Arabidopsis*. *Plant Physiol* 138:1046-1057.
46. Polge C, *et al.* (2009) Evidence for the existence in *Arabidopsis thaliana* of the proteasome proteolytic pathway. *J Biol Chem* 284:35412-35424.
47. Suter U, Heymach JV, Jr., & Shooter EM (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. *EMBO J* 10:2395-2400.
48. Chen Z-Y, *et al.* (2005) Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *J Neurosci* 25:6156-6166.
49. Geng Z, Xu F-Y, Huang S-H, & Chen Z-Y (2011) Sorting protein-related receptor SorLA controls regulated secretion of glial cell line-derived neurotrophic factor. *J Biol Chem* 286:41871-41882.
50. Conticello SG, *et al.* (2003) The prodomain of a secreted hydrophobic mini-protein facilitates its export from the endoplasmic reticulum by hitchhiking on sorting receptors. *J Biol Chem* 278:26311-26314.
51. Tian MY, Benedetti B, & Kamoun S (2005) A second kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato. *Plant Physiol* 138:1785-1793.
52. Hanai H, *et al.* (2000) Existence of a plant tyrosylprotein sulfotransferase: novel plant enzyme catalyzing tyrosine O-sulfation of preprophytosulfokine variants *in vitro*. *FEBS Lett* 470:97-101.
53. Meyer M, Leptihn S, Welz M, & Schaller A (2016) Functional characterization of propeptides in plant subtilases as intramolecular chaperones and inhibitors of the mature protease. *J Biol Chem* 291:19449-19461.

54. Cedzich A, *et al.* (2009) The protease-associated (PA) domain and C-terminal extension are required for zymogen processing, sorting within the secretory pathway, and activity of tomato subtilase 3 (SlSBT3). *J Biol Chem* 284:14068-14078.
55. Anderson ED, *et al.* (2002) The ordered and compartment-specific autoproteolytic removal of the furin intramolecular chaperone is required for enzyme activation. *J Biol Chem* 277:12879-12890.
56. Janzik I, Macheroux P, Amrhein N, & Schaller A (2000) *Le*SBT1, a subtilase from tomato plants. Overexpression in insect cells, purification and characterization. *J Biol Chem* 275:5193-5199.
57. Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol* 20:1203-1207.
58. Pédelacq JD, Cabantous S, Tran T, Terwilliger TC, & Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nature Biotech* 24:79-88.
59. Pross E, Soussoula L, Seitl I, Lupo D, & Kuhn A (2016) Membrane targeting and insertion of the C-Tail protein SciP. *J Mol Biol* 428:4218-4227.
60. Clough SJ & Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735-743.
61. Viotti C, *et al.* (2013) The endoplasmic reticulum is the main membrane source for biogenesis of the lytic vacuole in *Arabidopsis*. *Plant Cell* 25:3434-3449.
62. Lampropoulos A, *et al.* (2013) GreenGate - a novel, versatile, and efficient cloning system for plant transgenesis. *PLoS ONE* 8:e83043.

Figure legends:

Figure 1: Reduced gravicurvature of P_{CLEL6}:EPI1a, P_{CLEL9}:EPI1a and *tpst-1* seedlings is rescued by addition of mature CLEL6 or CLEL9 peptides. Gravicurvature of (A) three independent P_{CLEL6}:EPI1a and (C) P_{CLEL9}:EPI1a lines (gray bars) is significantly reduced in comparison to the wild type (white bars). Gravicurvature of P_{CLEL6}:EPI1a and P_{CLEL9}:EPI1a lines is restored to wild-type levels by application of (B) 10 nM CLEL6 or (D) 300 nM CLEL9 (hatched bars). (E) *tpst-1* gravicurvature in comparison to the wild-type control. (F) Gravicurvature of *tpst-1* seedlings treated (hatched bars) with CLEL6 (10 nM) or CLEL9 (300 nM) as compared to the untreated *tpst-1*control (gray bars). Seedlings were grown for five days in the dark on ½ MS medium with peptides added as indicated. Plates were rotated 90 ° and gravicurvature was assessed after two days as the angle of the hypocotyl with the horizontal. Panel AS was modified from ref. 35. Data are shown for one representative of at least two independent experiments as the mean ± SE ($n \geq 15$). *** indicates significant differences at $P < 0.001$ (two-tailed t test).

Figure 2: **Pre-processing of CLEL6 by SBT6.1 in the *cis*-Golgi is required for peptide maturation and secretion.** (A) Sequence of the CLEL6 precursor without the signal peptide. Proteolytic processing sites and other post-translational modifications are indicated, mature CLEL6 peptide sequence underlined. (B) Schematic representation of expression constructs used for subcellular localization studies. (C) Processing of the ER-restrained CLEL6 (KDEL) construct with (+) or without (-) BFA treatment, analyzed by anti-GFP immunoblotting. (E, F) Immunoblot analysis of ER-restrained (KDEL) and Golgi-localized (XylT) precursor processing compared to the secreted form (Sec) and the precursor lacking the two SBT6.1 cleavage sites (Sec^m). Fully and partially processed precursors are indicated by the red arrow head and asterisks, respectively. (D, G-H) Co-localization of the different expression constructs with ER (VMA12-mRFP) and Golgi (ManI-mCherry and ST-mCherry) markers analyzed by fluorescence microscopy. Pictures show an overlay of the green (500-550 nm) and red (610-670 nm) fluorescence channels. The dotted areas are shown in higher magnification in the insets. Scale bars represent 5 µm.

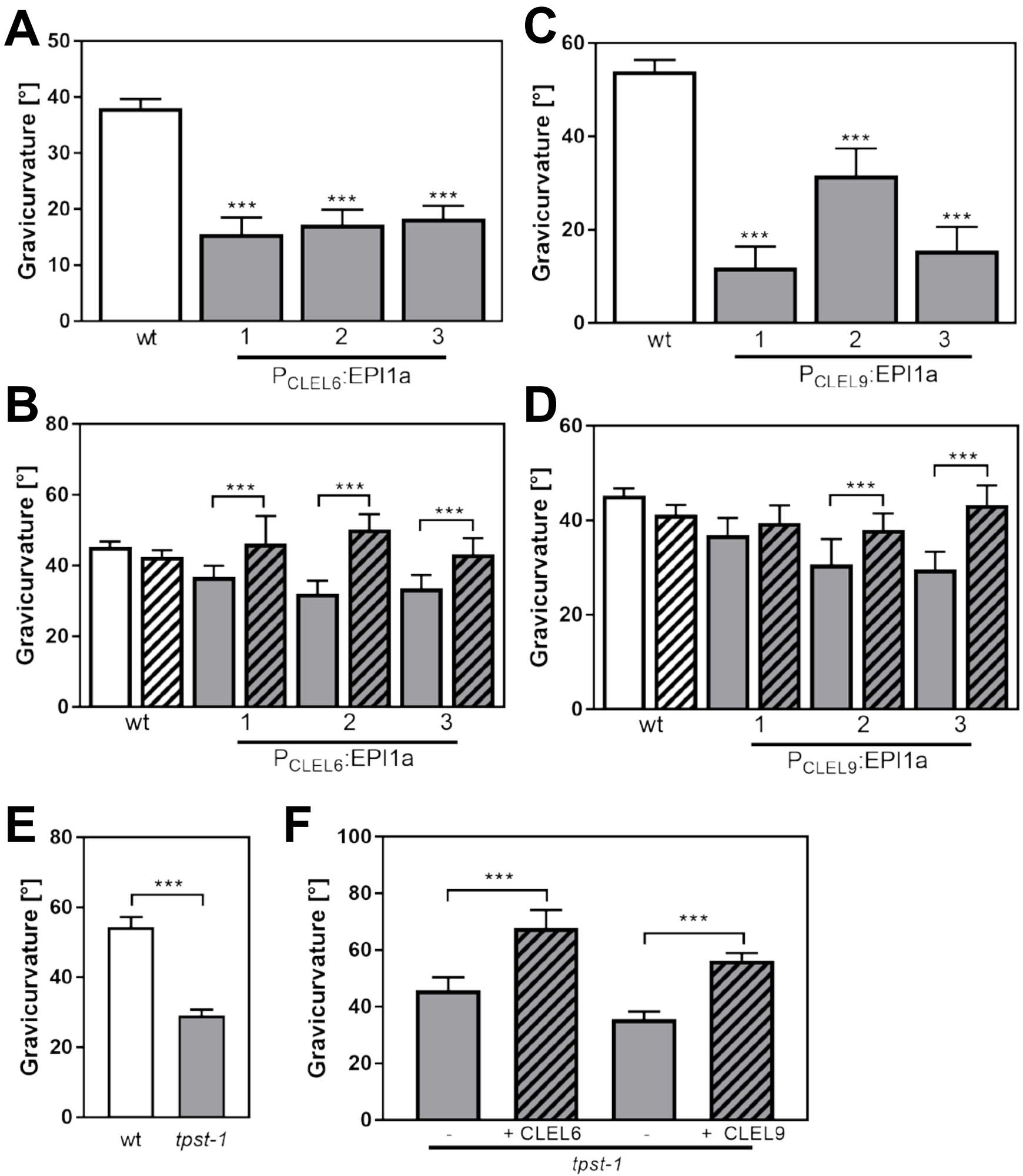
Figure 3: N-terminal maturation of CLEL6 occurs in a post-Golgi compartment. (A) Sequence and post-translational modification sites of Δ-CLEL6 constructs lacking the prodomain

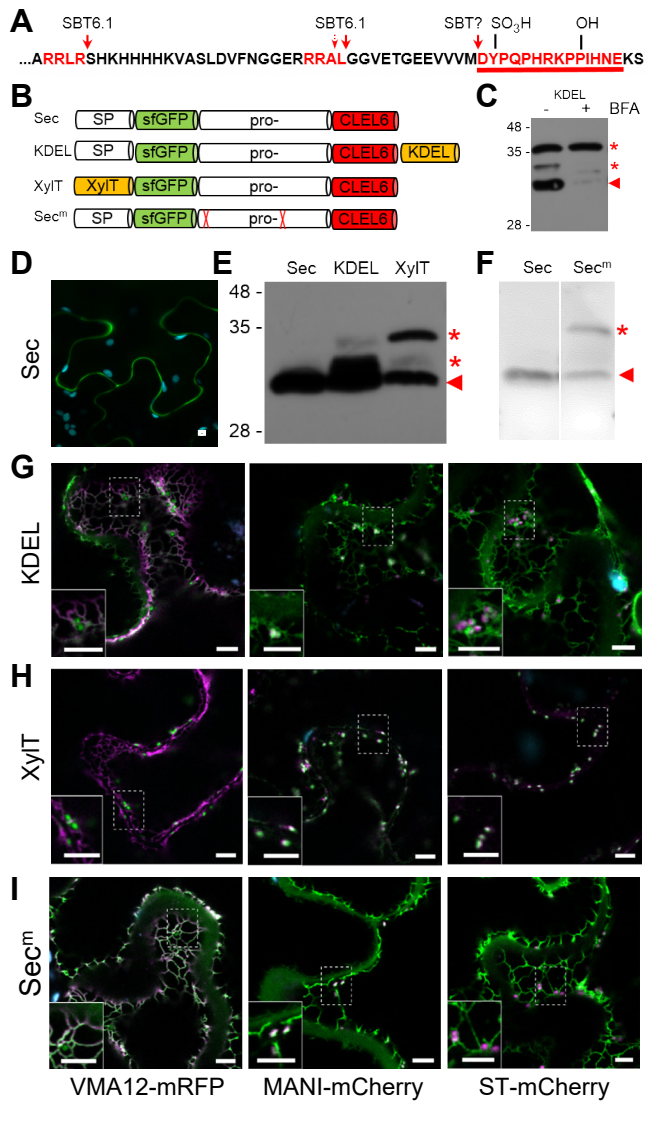
region encompassed by the two SBT6.1 cleavage sites; mature CLEL6 peptide sequence underlined. (B) Schematic representation of expression constructs used to localize the subcellular compartment of CLEL6 maturation. (C) Processing of the secreted (Δ-Sec), ER-restrained (Δ-KDEL) and Golgi-localized (Δ-XylT) constructs analyzed by anti-GFP immunoblotting. Fully and partially processed precursors are indicated by the red arrow head and asterisks, respectively. (D-F) Co-localization of the different expression constructs with ER (VMA12-mRFP) and Golgi (ManI-mCherry and ST-mCherry) markers analyzed by fluorescence microscopy. Pictures show an overlay of the green (500-550 nm) and red (610-670 nm) fluorescence channels. The dotted areas are shown in higher magnification in the insets. The white arrow marks apoplastic localization; scale bars represent 5 µm.

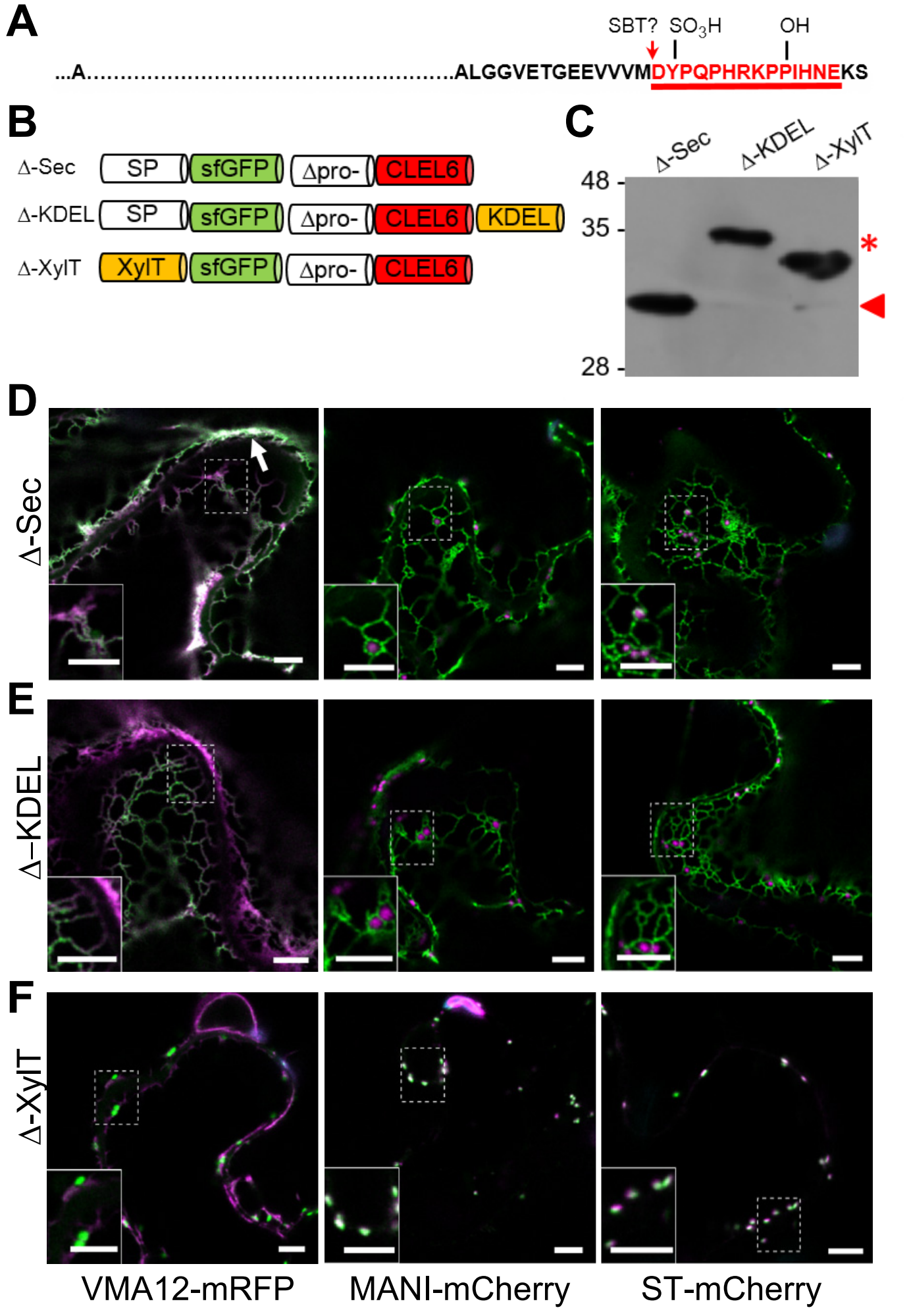
Figure 4: N-terminal maturation and the formation of bioactive CLEL6 and 9 peptides are aspartate-dependent (A, B) The relevance of the N-terminal aspartate for precursor processing was analyzed on anti-GFP immunoblots for the secreted (Δ-Sec) and Golgi-anchored (Δ-XylT) constructs by alanine substitution (D71A and D66A for the CLEL6 and CLEL9 precursors, respectively). (C) Representative pictures of seedlings that were grown for five days under control conditions, or treated with cell wall extracts from *N. benthamiana* plants expressing wild-type precursors of CLEL6 and CLEL9, or the corresponding D71A and D66A mutants. (D) Representative picture of a control root compared to a root grown on medium supplemented with CLEL6 peptide. (E) Changes in root growth direction [given as the mean of directional changes per root ± SD, n ≥59] for control seedlings (white bar) and seedlings treated as described in (C) (gray bars for CLEL6 and CLEL9 precursors; hatched bars for the D71A and D66A mutants). Data are shown for one representative out of three independent experiments.

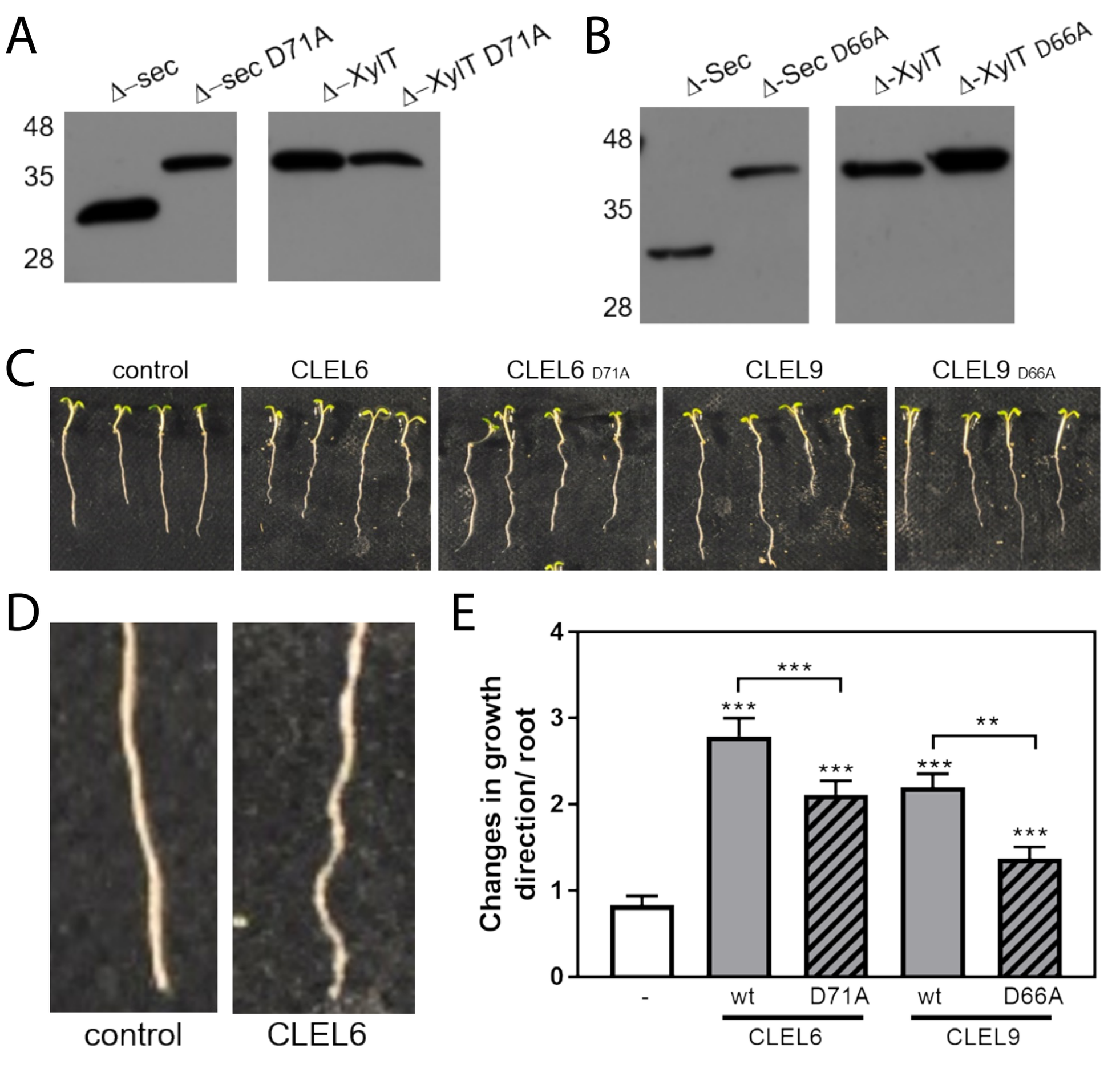
Figure 5: Maturation of the CLEL6 precursor in the secretory pathway. As a first processing step, the signal peptide is cleaved off upon entry into the ER. After exit from the ER, the precursor is processed by SBT6.1 in the cis-Golgi at two S1P cleavage sites within its variable prodomain. Still in the Golgi, the peptide moiety is tyrosine-sulfated by TPST, and proline hydroxylated. Candidate proline-4-hydroxylases have been identified, but it is still unclear which of these enzymes is responsible for peptide modification. There is also circumstantial evidence for proline-

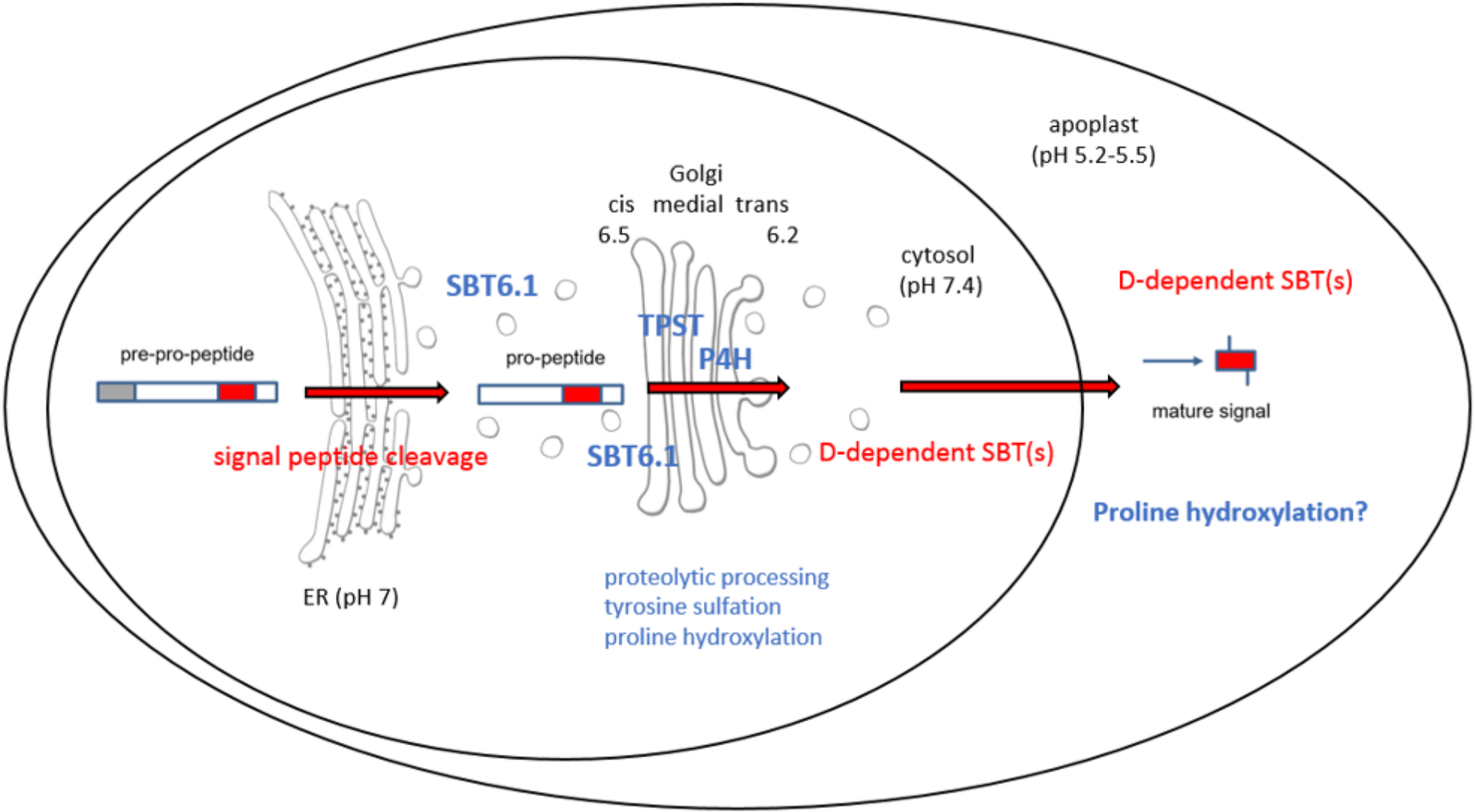
4-hydroxylase activity in the cell wall (36). N-terminal maturation by Asp-dependent SBTs occurs in a post-Golgi compartment.


A
Gravicurvature [°]
50
40
30
20
10
0
***
***
***
wt
1
2
3
P_CLEL6:EPI1a
C
Gravicurvature [°]
60
40
20
0
***
***
***
wt
1
2
3
P_CLEL9:EPI1a
B
Gravicurvature [°]
80
60
40
20
0
***
***
***
wt
1
2
3
P_CLEL6:EPI1a
D
Gravicurvature [°]
60
40
20
0
***
***
wt
1
2
3
P_CLEL9:EPI1a
E
Gravicurvature [°]
80
60
40
20
0
***
wt
tpst-1
F
Gravicurvature [°]
100
80
60
40
20
0
***
***
-
+ CLEL6
-
+ CLEL9
tpst-1


A
SBT6.1
SBT6.1
SBT?
SO3H
OH
...ARRLRSHKHHHHKVASLDVFNGGERRRALGGVETGEEVVVMDYPQPHRKPPIHNEKS
B
Sec
SP
sfGFP
pro-
CLEL6
KDEL
SP
sfGFP
pro-
CLEL6
KDEL
XylT
XylT
sfGFP
pro-
CLEL6
Secm
SP
sfGFP
pro-
CLEL6
C
KDEL
-
+
BFA
48
35
28
D
Sec
E
Sec
KDEL
XylT
48
35
28
F
Sec
Secm
G
KDEL
H
XylT
I
Secm
VMA12-mRFP
MANI-mCherry
ST-mCherry


A
SBT?
SO_3H
OH
...A.....ALGGVETGEEVVVMDYPQPHRKPPIHNEKS
B
Δ-Sec
SP
sfGFP
Δpro-
CLEL6
Δ-KDEL
SP
sfGFP
Δpro-
CLEL6
KDEL
Δ-XylT
XylT
sfGFP
Δpro-
CLEL6
C
Δ-Sec
Δ-KDEL
Δ-XylT
48
35
28
*
D
Δ-Sec
E
Δ–KDEL
F
Δ-XylT
VMA12-mRFP
MANI-mCherry
ST-mCherry


A
Δ–sec
Δ–sec D71A
Δ–XylT
Δ–XylT D71A
48
35
28
B
Δ-Sec
Δ-Sec D66A
Δ-XylT
Δ-XylT D66A
48
35
28
C
control
CLEL6
CLEL6 D71A
CLEL9
CLEL9 D66A
D
control
CLEL6
E
Changes in growth direction/ root
4
3
2
1
0
***
***
***
**
***
***
-
wt
D71A
wt
D66A
CLEL6
CLEL9


apoplast
(pH 5.2-5.5)
Golgi
cis medial trans
6.5
6.2
cytosol
(pH 7.4)
SBT6.1
TPST
P4H
D-dependent SBT(s)
pre-pro-peptide
pro-peptide
mature signal
signal peptide cleavage
SBT6.1
D-dependent SBT(s)
Proline hydroxylation?
ER (pH 7)
proteolytic processing
tyrosine sulfation
proline hydroxylation