## Tansley review

# From structure to function – a family portrait of plant subtilases

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Received: *1 December 2016*
Accepted: *13 March 2017*

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## Contents

## Summary

Subtilases (SBTs) are serine peptidases that are found in all three domains of life. As compared with homologs in other *Eucarya*, plant SBTs are more closely related to archaeal and bacterial SBTs, with which they share many biochemical and structural features. However, in the course of evolution, functional diversification led to the acquisition of novel, plant-specific functions, resulting in the present-day complexity of the plant SBT family. SBTs are much more numerous in plants than in any other organism, and include enzymes involved in general proteolysis as well as highly specific processing proteases. Most SBTs are targeted to the cell wall, where they contribute to the control of growth and development by regulating the properties of the cell wall and the activity of extracellular signaling molecules. Plant SBTs affect all stages of the life cycle as they contribute to embryogenesis, seed development and germination, cuticle formation and epidermal patterning, vascular development, programmed cell death, organ abscission, senescence, and plant responses to their biotic and abiotic environments. In this article we provide a comprehensive picture of SBT structure and function in plants.

## I. Introduction

The erection of anything new is usually preceded by the demolition of the old. Likewise, *de novo* protein synthesis is intricately linked to proteolytic degradation of proteins that are no longer needed, damaged, or misfolded and potentially harmful. In addition to this important housekeeping function, protein turnover is also a prerequisite for acclimatization, as accommodation to changing

environmental conditions may require proteomic adjustments. But there is more to proteases than protein turnover; they also serve important regulatory functions (Schaller, 2004; van der Hoorn, 2008). As proteolysis is essentially irreversible, it controls many aspects of plant growth, development and defense by selective degradation of regulatory proteins (Vierstra, 2009). Besides protein degradation, proteases are also responsible for the post-translational modification of other proteins by limited proteolysis at highly specific sites. Limited proteolysis may be required for protein assembly and subcellular targeting, and the specific processing of precursor proteins controls the activity of enzymes, regulatory proteins and signaling peptides (Schaller, 2004). This implies that the proteases involved are substrate-specific, and that their activity is tightly regulated, in both time and space (van der Hoorn, 2008).

Most proteases in plants belong to the catalytic class of serine peptidases. Among the serine peptidases, those related to bacterial subtilisin constitute the largest family (the subtilase (SBT) family). SBTs include enzymes for unselective protein degradation and others for specific precursor processing. They have been implicated in protein turnover, and in the regulation of growth and development (Schaller *et al.*, 2012). However, the specific function and the physiological substrates of SBTs and other regulatory proteases have long remained elusive in plants (van der Hoorn, 2008). Since the last comprehensive review of the plant SBT family (Schaller *et al.*, 2012), considerable progress has been made in these areas. We are now beginning to understand how SBTs execute their specific roles, and how their function *in vivo* relates to structure and biochemistry. These are the issues that will be addressed in this Tansley review.

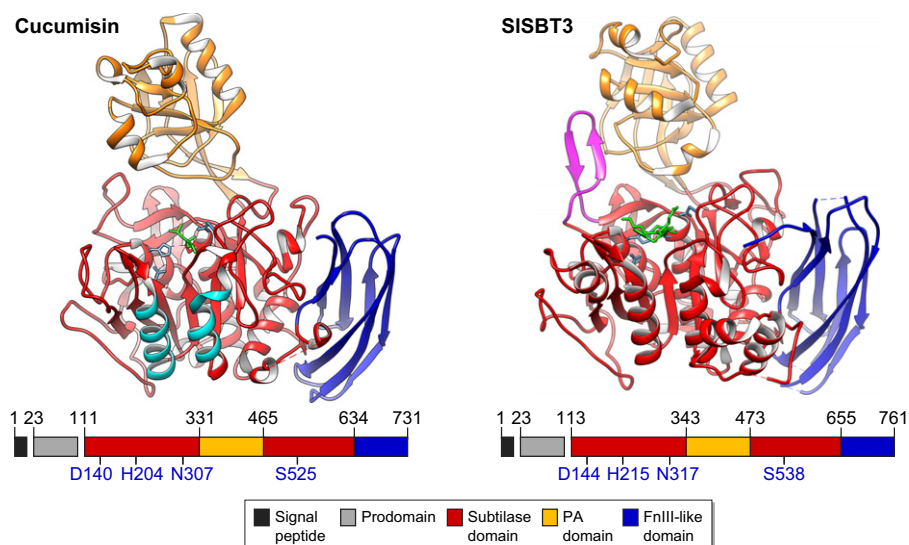## II. Biochemistry and structure of plant SBTs

Subtilases, the S8 peptidase family according to the classification in the MEROPS database (Rawlings *et al.*, 2010), constitute the second largest family of serine proteases. They derive their name from subtilisins found in the *Bacillus subtilis* group of Gram-positive bacteria, which are characterized by a specific arrange-ment of the Asp, His and Ser residues of the catalytic triad (Smith *et al.*, 1966), and by a unique structural fold consisting of a highly twisted, seven-stranded β-sheet sandwiched between two layers of α-helices (Wright *et al.*, 1969). Bacterial subtilisins are synthesized as preproenzymes with an amino terminal signal peptide for secretion, a prodomain that is autocatalytically cleaved during zymogen maturation, and the catalytic subtilisin domain. These features and the general preproprotein structure are shared between bacterial subtilisins and SBTs in plants and other eukaryotes (Schaller *et al.*, 2012). However, plant SBTs typically possess two additional domains including the protease-associated (PA) domain as a large insertion between the His and the Ser residues of the catalytic triad, and a C-terminal fibronectin (Fn) III-like domain. The function of the individual domains has been analyzed in detail for SlSBT3 from tomato (Cedzich *et al.*, 2009) and for cucumisin from melon fruits (Yamagata *et al.*, 1994), which can be regarded as prototypical members of the plant SBT family (Fig. 1).

The prodomain serves dual functions. First, it assists in folding and is thus required for enzyme maturation during passage through the secretory pathway. Prodomain deletion mutants do not pass endoplasmic reticulum (ER) quality control and are retained intracellularly (Cedzich *et al.*, 2009). Secretion is restored upon coexpression of the prodomain as a separate polypeptide chain *in trans* (Meyer *et al.*, 2016b). Prodomain function in plant SBTs thus resembles that of subtilisin propeptides, which have been described as intramolecular chaperones that are required for successful folding and secretion of the catalytic domain (Zhu *et al.*, 1989; Bryan, 2002). Second, the prodomain inhibits the activity of its own protease. After separation from the enzyme proper by autocatalytic cleavage (Cedzich *et al.*, 2009), the prodomain is not released but binds to the subtilisin domain with high affinity, forming an autoinhibited complex of exceptional stability (Nakagawa *et al.*, 2010; Meyer *et al.*, 2016b). Crystal structure analysis of the cucumisin/prodomain complex revealed the mode of interaction and enzyme inhibition. The C-terminus of the prodomain binds to the active site cleft in a substrate-like manner. Additional exosite interactions of the prodomain with two parallel surface helices of the protease contribute to complex formation (Fig. 1; Sotokawauchi *et al.*, 2017). In contrast to SlSBT3, which shows high specificity for its own prodomain (Meyer *et al.*, 2016b), cucumisin is also inhibited by distantly related prodomains of SBTs from rice and Arabidopsis (Nakagawa *et al.*, 2010). Considering the substrate-like binding of the prodomain to the active site of the protease, the higher substrate selectivity of SlSBT3 as compared with cucumisin offers an explanation for this difference in specificity. For the release of autoinhibition, the prodomain is cleaved autocatalytically in a strictly pH-dependent manner (Meyer *et al.*, 2016b). In addition, further trimming of the N-terminus in a second pH-dependent processing step may be required for enzyme maturation (Janzik *et al.*, 2000). These two factors, the stability of the complex on the one hand, and pH-dependence of prodomain cleavage and release from autoinhibition on the other, provide efficient means to keep the protease inactive until it reaches the acidic environment of the *trans* Golgi, and to prevent precocious activation in earlier compartments of the secretory pathway.

The PA domain is known from several classes of unrelated proteins, and has generally been described as a protein–protein interaction module (Mahon & Bateman, 2000). As such it is responsible for the binding of cargo proteins to plant vacuolar sorting receptors (Luo *et al.*, 2014) and for endosomal trafficking of PA-domain E3 ligases (Yamazaki *et al.*, 2013; van Dijk *et al.*, 2014); in proteases it contributes to substrate recognition and may control access to the active site (Bruinenberg *et al.*, 1994; Luo & Hofmann, 2001; Kagawa *et al.*, 2009; Kurata *et al.*, 2010). Interacting with the substrate, particularly with substrate residues on the prime side (i.e. downstream) of the cleaved peptide bond, the PA domain also contributes to substrate selectivity in plant SBTs (Murayama *et al.*, 2012; Tan-Wilson *et al.*, 2012). The residue two positions downstream of the substrate's scissile bond (P2′) interacts with $Arg_{433}$ in the PA domain of SlSBT3 (Tan-Wilson *et al.*, 2012), explaining its strong preference for

**Fig. 1** Structure of SlSBT3 and cucumisin. The tertiary structure is shown for cucumisin on the left (Protein Data Bank (PDB) code 3VTA) and SlSBT3 on the right (PDB code 3I74). Covalently bound inhibitors are shown in green. The side chains of the active-site Asp, His, and Ser residues are shown in sky blue. The salient β-hairpin of SlSBT3 is highlighted in magenta, and the two surface helices that interact with the prodomain of cucumisin are in cyan. Figures were generated using UCSF Chimera (Pettersen *et al.*, 2004). Schematic representations of the primary structure are shown below. Numbers label amino acids at the domain junctions and active site residues. The color scheme for the different domains is indicated.

negatively charged Glu in this position (Meyer *et al.*, 2016b). In soybean C1 protease, on the other hand, interaction of the PA domain with the P4′ residue determines its preference for longer peptide substrates (Tan-Wilson *et al.*, 2012). Extended substrate recognition motives that include prime-side residues distinguish plant SBTs from bacterial subtilisins, which lack the PA domain and thus depend on nonprime-side residues for substrate recognition (Tan-Wilson *et al.*, 2012). The PA domain thus contributes to increased substrate selectivity in plant SBTs as compared with bacterial subtilisins. In SlSBT3 the PA domain serves an additional role as it mediates homodimerization as a prerequisite for enzyme activation. Within the SlSBT3 dimer, the PA domain of one protomer interacts with a prominent β-hairpin of the other to keep the active site accessible for substrates (Ottmann *et al.*, 2009; Fig. 1). Structural modeling of representative Arabidopsis SBTs indicated that the β-hairpin is not conserved in the entire family and, therefore, this autoregulatory mechanism is unlikely to be operating in all plant SBTs (Rose *et al.*, 2010).

The C-terminal FnIII-like domain, on the other hand, is a more general feature of plant SBTs and is functionally required for some (e.g. SlSBT3; Cedzich *et al.*, 2009) but not all SBTs (e.g. cucumisin; Yamagata *et al.*, 1994). In SlSBT3, it is clipped onto the catalytic domain by its extreme C-terminus which inserts into a hydrophobic pocket close to the substrate binding channel, thus stabilizing the loop system near the active site (Schaller, 2013). Further contributing to the stability of the enzyme, the FnIII-like domain shields hydrophobic surface patches from solvent (Ottmann *et al.*, 2009). The extraordinary thermostability of SlSBT3 and cucumisin is all the more remarkable given that both enzymes lack calcium (Ottmann *et al.*, 2009; Murayama *et al.*, 2012). Outside the plant kingdom, stability of SBTs depends on the binding of several calcium ions which contribute their binding energy to the overall free energy of folding (Alexander *et al.*, 2001). It thus seems that evolution has taken a different path toward stabilization of the subtilisin fold in plants as compared with other organisms.

## III. Phylogeny of plant SBTs and family organization

The MEROPS database distinguishes two SBT subfamilies, S8B with kexin from the yeast *Saccharomyces cerevisiae* as the type example, and S8A which comprises subtilisin Carlsberg from *Bacillus licheniformis* as the prototype, as well as thermitase, lantibiotic leader peptidase, proteinase K and pyrolysin-like enzymes as additional homology groups (Siezen & Leunissen, 1997). Kexins are well known in mammals for their role as proprotein convertases (PCs), which are involved in the specific processing of peptide hormone precursors, receptor proteins, viral surface proteins and bacterial toxins at basic cleavage sites (Steiner, 1998; Seidah *et al.*, 2013). Seven kexin-like PCs are found in the S8B subfamily in mammals, in addition to two pyrolysins and proteinase K in S8A. Plants do not have kexins; in lieu thereof the S8A subfamily is largely expanded, comprising 56 pyrolysin-related enzymes in *Arabidopsis thaliana*. The six SBT subgroups that were distinguished by Rautengarten *et al.* (2005) are largely supported by phylogenetic analysis and gene structure (Fig. 2; Supporting Information Fig. S1). The two most distantly related enzymes, AtSBT6.1 and AtSBT6.2, are the orthologs of mammalian site-1-protease (S1P) and tripeptidyl peptidase II (TPP2), respectively, while all other family members in subgroups SBT1 to SBT5 appear to be plant-specific. Large *SBT* gene families have also been identified in other plant species, comprising 63 pyrolysin genes in rice (*Oryza sativa*; Tripathi & Sowdhamini, 2006), 82 in grape (*Vitis vinifera*; Cao *et al.*, 2014; Figueiredo *et al.*, 2016), and 82 in potato (*Solanum tuberosum*; Norero *et al.*, 2016). As compared with the entire genome of Arabidopsis, 60% of which is contained in large duplicated segments, *SBT* family expansion has been driven predominantly by tandem gene duplications. The majority of *SBT* genes in Arabidopsis and grape are found in tandem gene clusters, the most prominent example being 13 members tandemly arrayed on grape chromosome 13 (Rautengarten *et al.*, 2005; Cao *et al.*, 2014). These 13 genes form a single phylogenetic clade, suggesting that they arose from recent tandem duplications (Cao *et al.*, 2014). Only 16 orthologous pairs were identified in a comparison of *SBT*
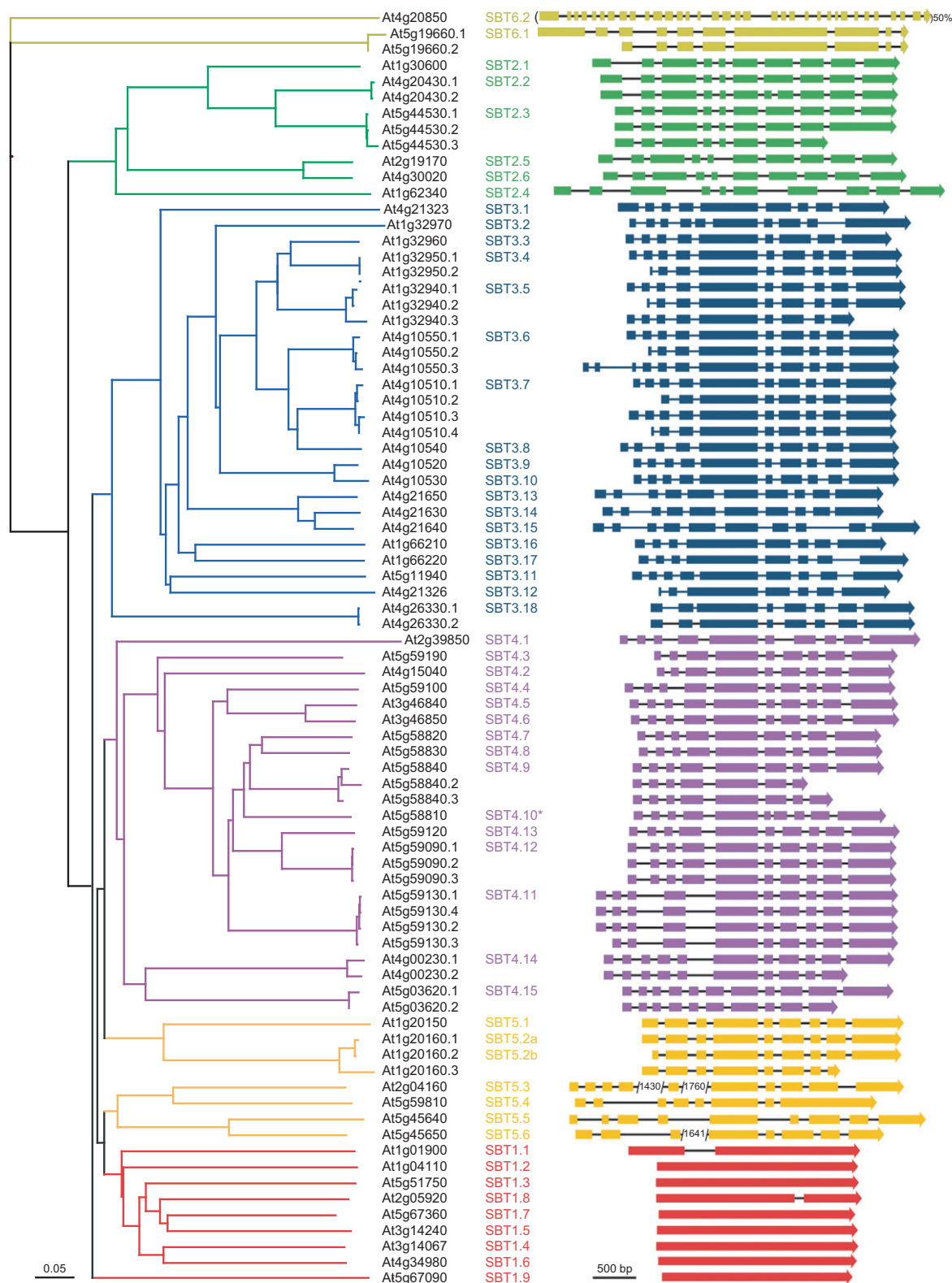
**Fig. 2** Phylogenetic tree of Arabidopsis subtilases (SBTs). Amino acid sequences of Arabidopsis SBTs as annotated in the latest The Arabidopsis Information Resource (TAIR) release (TAIR 10) were aligned with CLUSTALX using the default parameter settings. The phylogenetic tree was obtained with the neighbor-joining method using SBT6.1 and 6.2 as an outgroup, and it was visualized using TREEVIEW. Different gene models as a result of alternative splicing or alternative start codons are numbered consecutively as AtXgXXXXX.1, .2, etc. Different colors are used to distinguish the SBT subfamilies 1–6. Exon–intron structure is shown at the indicated scale, except for SBT6.2, which had to be reduced in size by 50%. The length of very large introns is indicated in base pairs (bp). SBT4.10 (marked by an asterisk) was included here even though it may be a pseudogene and is no longer shown in TAIR 10.

families between rice and Arabidopsis, while more often several genes in one species collectively are orthologs of a single gene in the other. Similarly, 17 *SBT* genes in Arabidopsis (*SBT3.1- SBT3.17*) form a clade that is orthologous to a single potato gene (P0001177G1000585) and, vice versa, *AtSBT1.9* is the ortholog of four *SBTs* in potato. Such unequal distribution of individual clades indicates that these genes evolved as a result of gene duplication events subsequent to speciation (Tripathi & Sowd-hamini, 2006; Norero *et al.*, 2016). Positive selection that was shown to act on some critical sites of grape *SBTs* may then have contributed to functional diversification of paralogous sequences (Cao *et al.*, 2014). These observations suggest that the *SBT* family was shaped in different species by evolutionary forces that are specific for their respective ecological niche, and we may thus predict that many SBTs are involved in processes related to the interaction of plants with their biotic and abiotic environments.

## IV. Physiological roles of plant SBTs

Our knowledge of SBT function in a physiological context is most advanced in Arabidopsis, and all functionally characterized Arabidopsis SBTs that will be covered in this article are compiled in Table 1. SBTs of known function in other plant species will also be included in the discussion.

### 1. Orthologs of mammalian SBTs

Site-1-protease and TPP2 are the two mammalian pyrolysins. AtSBT6.1 and 6.2 have been identified as their orthologs in Arabidopsis. These enzymes are involved in processes that are conserved across kingdoms. TPP2 is the only known exopeptidase among SBTs catalyzing the release of tripeptides from the N-terminus of its oligopeptide substrates. It also differs from other plant SBTs with respect to its cytoplasmic/nuclear localization. TPP2 consists of two polypeptides, both derived from the AtSBT6.2 precursor, that assemble into a large oligomeric complex (Book *et al.*, 2005). In mammals, TPP2 is mainly involved in general protein turnover and in the formation of peptides for MHC class I antigen presentation (Tomkinson & Lindås, 2005). Likewise in Arabidopsis, TPP2 (AtSBT6.2) acts downstream of the proteasome in a proteolytic pathway for the degradation of proteins that are damaged after heavy metal exposure (Polge *et al.*, 2009). The lack of any obvious phenotype for the *Atsbt6.2* mutant indicates that TPP2 function is not essential for plant growth and development under controlled conditions (Book *et al.*, 2005).

Site-1-protease (AtSBT6.1) is involved in the unfolded protein response (UPR) which contributes to a sophisticated quality control system in the ER of both animals and plants. It initiates the transduction of stress signals from the ER to the nucleus by activation of membrane-anchored transcription factors, activating transcription factor 6 (ATF6) in mammals, and bZIP28 in Arabidopsis (Liu & Howell, 2010). When unfolded proteins accumulate, ER-resident bZIP28 moves to the Golgi where it is cleaved by S1P (AtSBT6.1) and the metalloprotease S2P. Thereby, the N-terminal bZIP domain is released and translocates to the nucleus where it regulates UPR gene expression (Liu *et al.*, 2007a;

Che *et al.*, 2010; Liu & Howell, 2010). The same mechanism leads to the activation of a second membrane-bound transcription factor, bZIP17, which is cleaved by AtSBT6.1 as part of the salt stress response (Liu *et al.*, 2007b).

In addition to the membrane-anchored transcription factors that are cleaved in a S1P-like manner, pectin methylesterases (PMEs) and the precursors of several peptide growth factors have been identified as further substrates of AtSBT6.1. The inhibitory prodomain of group II PMEs is responsible for retention within the Golgi. Prodomain cleavage by AtSBT6.1 is thus required for the release and secretion of the mature enzyme (Wolf *et al.*, 2009). PME prodomains and bZIP transcription factors are processed by AtSBT6.1 at the carboxy-side of typical S1P cleavage sites (R[R,K] XL). Similar sites are also found in the precursors of the signaling peptides R(apid) AL(kalinization) F(actor) 23 (RRIL; Srivastava *et al.*, 2009) and GOLVEN1 (RRLR, RRAL; Ghorbani *et al.*, 2016), and these sites are also processed by AtSBT6.1. While cleavage by AtSBT6.1 appears to be required for RALF23 and GOLVEN1 function, it does not yield the bioactive peptides. Therefore, additional as yet unidentified proteases are implicated in peptide maturation.

### 2. Plant SBTs in biotic interactions

**Subtilases as modulators of plant immune responses**  The first plant SBT cloned from a higher plant and characterized as being involved in plant defense was P69, an extracellular protease from tomato plants (Vera & Conejero, 1988; Vera *et al.*, 1989; Tornero *et al.*, 1996a). P69 was characterized initially as a pathogenesis-related (PR) protein (PR-7) playing a role in pathogen defense, whose expression and accumulation were significantly enhanced in pathogen-infected tomato plants. P69 was later found to comprise different SBTs of *c.* 69 kDa in size (P69A–P69F) with their encoding genes arranged in clusters in the tomato genome (Tornero *et al.*, 1997; Jordá *et al.*, 1999, 2000; Meichtry *et al.*, 1999). Two of these protease genes, *P69B* and *P69C*, are coordinately and systemically induced by pathogen infection and salicylic acid treatment (Jordá & Vera, 2000; Jordá *et al.*, 2000). Tomato P69C was found to process an extracellular matrix-associated leucine-rich repeat protein (LRP), the first physiological substrate for an extracellular SBT identified in plants (Tornero *et al.*, 1996b). This processing of LRP by a P69 SBT was speculated to mediate molecular recognition to initiate immune signaling.

More recently, such a role was confirmed for AtSBT3.3, the ortholog of tomato P69C in Arabidopsis (Ramírez *et al.*, 2013). AtSBT3.3 was found in a genetic screen aiming at the identification of factors that suppress constitutive expression of the P69C::GUS transgene in the pathogen-resistant *constitutive subtilisin3* (*csb3*) mutant background (Gil *et al.*, 2005). Interestingly, the overexpression of *AtSBT3.3* results in enhanced innate immune responses, while its loss of function in *Atsbt3.3* mutants compromises them. *AtSBT3.3* expression initiates a durable autoinduction mechanism that involves chromatin remodeling and the activation of SA-dependent priming of defense genes for an amplified response. Moreover, the expression of *AtSBT3.3* sensitizes plants for enhanced activation of mitogen-activated protein (MAP)

**Table 1** Compilation of Arabidopsis subtilases (SBTs) discussed in this article

| Locus ID | Name | Alias | Function | Substrate | References |
|---|---|---|---|---|---|
| At1g01900 | AtSBT1.1 | | Callus induction | PSK4 | Srivastava *et al.* (2008) |
| | | | Embryo development | Unknown | D'Erfurth *et al.* (2012) |
| At1g04110 | AtSBT1.2 | SDD1 | Stomata development | Unknown | Berger & Altmann (2000) and von Groll *et al.* (2002) |
| At3g14067 | AtSBT1.4 | SASP | Reproductive development | Unknown | Martinez (2015) |
| At5g67370 | AtSBT1.7 | ARA12 | Mucilage release | Unknown | Rautengarten *et al.* (2008) |
| At1g62340 | AtSBT2.4 | ALE1 | Cuticle development | Unknown | Tanaka *et al.* (2001) and Xing *et al.* (2013) |
| At1g32960 | AtSBT3.3 | | Immune priming | Unknown | Ramírez *et al.* (2013) |
| At1g32940 | AtSBT3.5 | | Root development | PME17 | Senechal *et al.* (2014) |
| At4g21630 | AtSBT3.14 | | Nematode resistance | Unknown | Lozano-Torres *et al.* (2014) |
| At5g59090 | AtSBT4.12 | | Abscission | IDA | Schardon *et al.* (2016) |
| At5g59120 | AtSBT4.13 | | Abscission | IDA | Schardon *et al.* (2016) |
| At1g20160 | AtSBT5.2a | CRSP | Stomata development, | EPF2, IDA | Engineer *et al.* (2014), Schardon *et al.* (2016) |
| | AtSBT5.2b | | Abscission, | MYB30 | and Serrano *et al.* (2016) |
| | | | Attenuation of defense gene expression | | |
| At2g04160 | AtSBT5.3 | AIR3 | Lateral root development | Unknown | Veth-Tello (2005) |
| At5g59810 | AtSBT5.4 | | Meristem maintenance | Unknown | Liu *et al.* (2009) |
| At5g19660 | AtSBT6.1 | AtS1P | Precursor processing | bZIPs, PMEs, RALFs, GLV1 | Liu *et al.* (2007a,b), Srivastava *et al.* (2009), Wolf *et al.* (2009), Liu & Howell (2010), Ghorbani *et al.* (2016) and Stegmann *et al.* (2017) |
| At4g20850 | AtSBT6.2 | TPP2 | Protein turnover | Nonspecific | Book *et al.* (2005), Polge *et al.* (2009) and Ghorbani *et al.* (2016) |

kinases following pathogen attack. Conversely, the pathogen-induced expression of SA-dependent defense genes and MAP kinase activation are reduced in the *Atsbt3.3* mutant. Likewise, chromatin remodeling of defense-related genes was also impaired in *Atsbt3.3*, further supporting the importance of the protease for the establishment of immune priming. Although the extracellular target(s) of AtSBT3.3 remain to be identified, these data highlight the P69C/AtSBT3.3 SBT as a major regulator of primed immunity (Fig. 3a).

A recent study identified AtSBT5.2 as an atypical Arabidopsis SBT that is involved in the transcriptional regulation of defense responses by a mechanism previously undescribed in eukaryotes (Serrano *et al.*, 2016). *AtSBT5.2* is alternatively spliced, producing transcripts for two alternative proteins: the regular secreted protease AtSBT5.2(a) and the noncanonical intracellular SBT AtSBT5.2(b) (Fig. 3b). Retention of the first intron of *AtSBT5.2* results in AtSBT5.2(a), while the fully spliced transcript leads to a truncated protein that is subject to N-terminal myristoylation. Myristoylated AtSBT5.2(b) is targeted to endosomal vesicles, where it is able not only to interfere with endosomal trafficking but also to interact with the transcription factor MYB30 (Serrano *et al.*, 2016). MYB30 is a positive regulator of pathogen defense and hypersensitive cell death (HR)-associated responses (Raffaele & Rivas, 2013). AtSBT5.2(b)-mediated nuclear exclusion of MYB30 thus interferes with the defense gene-inducing activity of the transcription factor, resulting in a suppression of HR and impaired resistance, in a process that is independent of SBT catalytic activity (Serrano *et al.*, 2016; Fig. 3b).

By contrast, a barley protease that was recently shown to be involved in the production of thionins, clearly depends on its catalytic activity for its function in plant defense. Thionins are plant-specific antimicrobial peptides that are processed from larger precursor proteins (Bohlmann *et al.*, 1988). The thionin-processing protease enzyme (TPPE) of barley was identified as a SBT (accession no. BAJ93208; Plattner *et al.*, 2015). However, whether the processing activity is required for disease resistance remains to be shown. A contribution to insect resistance was demonstrated for SlSBT3 in tomato. Transgenic plants silenced for *SlSBT3* expression are impaired in the systemic induction of defense genes after wounding, and resistance against insect caterpillars is reduced (Meyer *et al.*, 2016a). The data support a role for SlSBT3 in systemic wound signaling, but its mechanism of action and physiological substrates remain to be identified. This is also the case for AtSBT3.14, which was found to be required for basal immunity to cyst nematodes in Arabidopsis. The expression of *AtSBT3.14* is strongly down-regulated in response to venom allergen-like proteins, which are part of the effector repertoire of plant-parasitic nematodes, and resistance against *Heterodera schachtii* is impaired in *AtSBT3.14* loss-of-function mutants (Lozano-Torres *et al.*, 2014). Direct interaction with a pathogen effector was shown for GbSBT1 from cotton (*Gossypium barbadense*). The protease was shown to interact with a prohibitin (PHB)-like effector protein which is secreted by *Verticillium dahliae* during infection (Duan *et al.*, 2016). Gain- and loss-of-function studies confirmed a role for GbSBT1 in *Verticillium* wilt resistance (Duan *et al.*, 2016). Yet, the proposed link between the GbSBT1/PHB interaction and the resistance phenotype remains to be established.

In the earlier examples, plant defense is generally improved and resistance enhanced by SBT activity. Interestingly, this weapon in the defensive arsenal of plants was turned upon themselves by phytopathogenic fungi in the genus *Colletotrichum*. Approximately
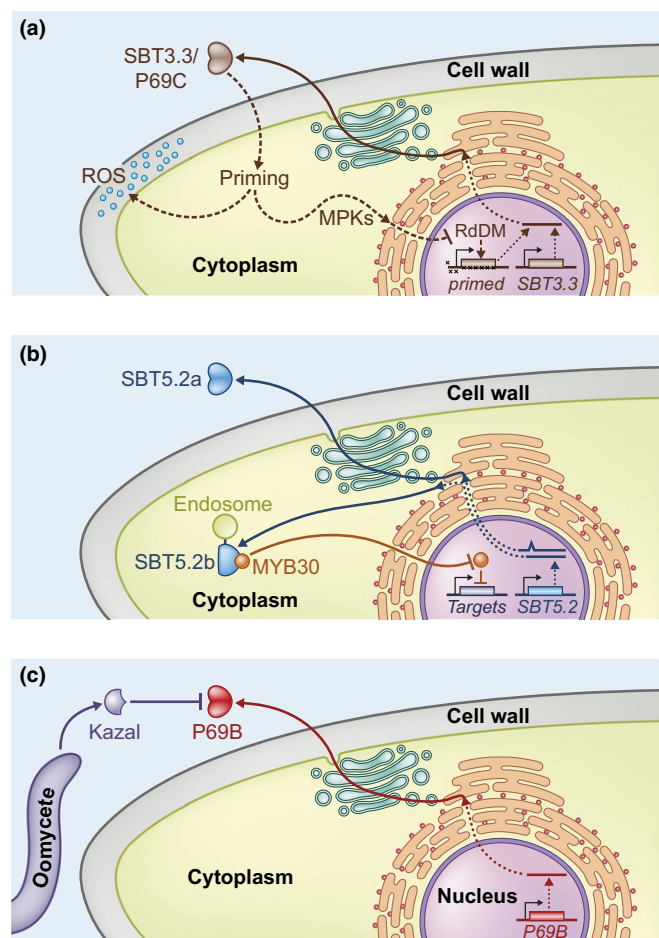
**Fig. 3** Different roles for subtilases (SBTs) in biotic interactions. (a) Mechanism of SBT3.3 self-activation and expression maintenance during immune priming. Pathogen perception triggers expression of SBT3.3/P69C. Once expressed and secreted to the apoplast, SBT3.3/P69C activates an MAP kinase-mediated signaling loop that negatively regulates epigenetic RNA-mediated DNA methylation (RdDM). As RdDM keeps defense-related genes silent in plants before pathogen encounter, the blocking of RdDM primes defense-related genes, including SBT3.3, for enhanced activation upon pathogen attack (Ramírez *et al.*, 2013). Overexpression of SBT3.3 in transgenic plants activates this positive self-regulatory loop that maintains RdDM compromised and defense genes primed. SBT3.3 expression also sensitizes plants for enhanced expression of the OXI1 kinase, reactive oxygen species (ROS) production, and mitogen-activated protein (MAP) kinase activation following pathogen attack. SBT3.3 is thus a key player in memory-based plant immunity and induced resistance. (b) The atypical subtilase SBT5.2(b) attenuates transcriptional activation of defense. Retention of an intron in *AtSBT5.2* results in a canonical secreted subtilase, SBT5.2(a), that was previously shown to regulate stomatal density under high $CO_2$ conditions (Engineer *et al.*, 2014). Alternative splicing leads to SBT5.2(b) which is N-terminally myristoylated and targeted to endosomal vesicles where it retains the defense-related transcription factor MYB30. Nuclear exclusion of MYB30 impairs target gene expression and, therefore, attenuates defense-related hypersensitive cell death (Serrano *et al.*, 2016). (c) Inhibition of P69B(-like) SBTs by Kazal inhibitors from oomycetes. Tomato P69B is an extracellular pathogenesis-related (PR) protein that is targeted by the Kazal-like inhibitors EPI1 and EPI10 secreted by the oomycete potato late blight pathogen *Phytophthora infestans*. Kazal-like inhibitors are common to oomycete pathogens of plants, and presumably target similar, P69B-like secreted defense-related host SBTs. Dotted and solid lines indicate transport of transcripts and proteins, respectively, whilst dashed lines indicate regulatory interactions.

150 million years ago, the *Colletotrichum* lineage acquired a plant SBT gene by horizontal gene transfer (Armijos Jaramillo *et al.*, 2013b; Gan *et al.*, 2013). Consistent with a role as a virulence factor, the plant-like SBT of *C. graminicola* is up-regulated during infection of maize, concomitant with the down-regulation of several host SBTs (Armijos Jaramillo *et al.*, 2013a). Direct evidence for a virulence function of plant-like SBTs in *Colletotrichum* is still missing, however. We conclude that the functions of plant SBTs in immune responses are manifold, ranging from immune priming to the regulation of defense gene expression, the generation of antimicrobial peptides, and the recognition or processing of pathogen effectors. In most cases, however, the mode of SBT action and their physiological substrates remain to be discovered.

**Subtilases as modulators of beneficial interactions with microorganisms** In addition to the contribution of SBTs to the regulation of plant immune responses, other studies have highlighted the involvement of SBTs in the interaction of plants with symbiotic microbes. A role for SBTs in mutualistic interactions was first reported for actinorhizal nodule formation in *Alnus glutinosa* (Ribeiro *et al.*, 1995). Expression levels of the SBT-encoding gene *Ag12* are markedly increased in cortical cells of the prefixation zone of actinorhizal nodules during early infection stages, and *Ag12* was thus proposed as an early nodulin gene involved in nodule development. The timing of *Ag12* expression further suggested that the protease is probably involved in protein processing rather than protein degradation (Ribeiro *et al.*, 1995). Similarly, the *Ag12* homolog *Cg12* of *Casuarina glauca* is induced in infected root hairs and nodule cortical cells in the very early stages after infection with Frankia. *Cg12* expression decreases with the establishment of nitrogen fixation and was not observed after infection with noninvasive strains, or ecto- or endomycorrhizal fungi (Svistoonoff *et al.*, 2003). Based on the specific induction of *Cg12* at early infection stages, it was suggested that the protease may be involved in the degradation of cell wall-associated proteins or in the maturation of a polypeptide in the signaling cascade triggered by Frankia infection (Svistoonoff *et al.*, 2003).

Several *SBT* genes are induced in *Lotus japonicus* during arbuscular mycorrhiza (AM) development after infection with *Glomus interradices* (*SbtM1*, *SbtM3*, *SbtM4* and *SbtS*), or during nodule formation after infection with the symbiotic bacterium *Mesorhizobium loti* (*SbtM4* and *SbtS*) (Kistner *et al.*, 2005; Takeda *et al.*, 2009). AM-induced expression of *SbtM1*, *SbtM3* and *SbtM4* depends on a signaling pathway that is common to both symbioses, whilst *SbtS* is regulated by an independent pathway (Takeda *et al.*, 2011). Loss-of-function studies confirmed a role for *SbtM1* and *SbtM3* during the development of intracellular infection structures for arbuscule development. Potential substrates of *SbtM1* and *SbtM3* include extracellular proteins from both fungus and plant that are present in the periarbuscular space (Takeda *et al.*, 2009).

**Subtilases as pathogen targets** The examples provided earlier build a strong case for the important role of SBTs in modulating plant immune responses as well as cell-to-cell signaling during symbiosis. This notion is further supported by the observation that pathogens target apoplastic SBTs in an attempt to suppress host

defense. The interaction of pathogen effectors with plant proteases at the plant–pathogen interface thus constitutes evidence for the important role played by apoplastic proteases during plant–pathogen coevolution (Dong *et al.*, 2014; Ilyas *et al.*, 2015).

The inhibition of SBT activity by pathogen-derived Kazal-like proteinase inhibitor has been described for several host–pathogen interactions in both plants and animals. The Kazal family consists of small (5 kDa) serine protease inhibitors classified as typical or atypical, based on the number of disulfide bonds. While typical Kazal inhibitors also inhibit other serine proteases, mainly trypsin-like enzymes (Morris *et al.*, 2002; Lai *et al.*, 2016; Pariani *et al.*, 2016), atypical Kazals are more specific for SBTs (Tian & Kamoun, 2005). Kazal-like inhibitors secreted by the apicomplexan parasite *Toxoplasma gondii* inhibit host-derived SBTs, possibly contributing to colonization (Morris *et al.*, 2002; Pszenny *et al.*, 2002; Morris & Carruthers, 2003). The genome of *Phytophthora infestans*, a devastating oomycete pathogen of potato and tomato, codes for 33 Kazal-like protease inhibitors, two of which were functionally characterized: Extracellular Protease Inhibitor (EPI) 1 and EPI10 (Tian *et al.*, 2004, 2005; Tian & Kamoun, 2005). The expression of *EPI1* increases during infection of tomato leaves and the protein was shown to interact with the host protease P69B (Fig. 3c). Recombinant EPI1 inhibits P69B and subtilisin-A but not trypsin or chymotrypsin, suggesting specificity towards S8 serine proteases (Tian *et al.*, 2004). S8 specificity is mediated by the atypical Kazal-domain (EPI1a) of the two-headed EPI1 inhibitor (Tian *et al.*, 2004; Tian & Kamoun, 2005). EPI10, comprising one atypical and two typical Kazal domains, is also expressed during infection. Similar to EPI1, EPI10 interacts with P69B and inhibits apoplastic SBTs of tomato (Tian *et al.*, 2005) and Arabidopsis (Schardon *et al.*, 2016). The finding that *P. infestans* targets apoplastic SBTs using at least two independent multidomain Kazal inhibitors corroborates a role of these proteases in tomato plant defense (Fig. 3c).

In the last few years, aided by the advances in genome sequencing, several research groups have reported the existence of Kazal-like inhibitors in other plant pathogenic oomycetes, including *P. ramorum*, *P. palmivora*, *Plasmopara halstedii* and *Pythium ultimum* (Meijer *et al.*, 2006; Cheung *et al.*, 2008; Chinnapun *et al.*, 2009; Sharma *et al.*, 2015). However, these inhibitors remain to be functionally characterized, and genetic data supporting their role in virulence are still scarce. It is worth noting that Kazal-like inhibitors are not present in the genomes of fungal and bacterial plant pathogens (Tian & Kamoun, 2005). Given that these pathogens also populate the apoplast and are thus exposed to secreted P69B-like defense proteases, it is tempting to speculate that they have their own SBT inhibitors, different from the Kazal family, that still await discovery.

## 3. Plant SBTs as regulators of cell death

Given the large number of SBT family members in each plant species, it is probably not surprising that some of these enzymes also were found to be engaged in programmed cell death (PCD). What is surprising is the substrate specificity of cell death-related SBTs. These proteases are unusual with respect to their strict aspartate

specificity of hydrolysis. Substrate specificity is very similar to that of animal cysteine-dependent death proteases, caspases, and hence they were called phytaspases (Chichkova *et al.*, 2010). Like caspases, phytaspases recognize several amino acid residues preceding the scissile peptide bond of their substrates, which confers exclusive specificity to phytaspase-mediated hydrolysis. However, unlike caspases, the preferred phytaspase recognition motif is remarkably hydrophobic (Galiullina *et al.*, 2015).

Phytaspases, like other plant SBTs, are synthesized as inactive preproenzymes, in which the signal peptide guides entry into the secretory pathway, where the prodomain is autocatalytically cleaved (Cedzich *et al.*, 2009; Chichkova *et al.*, 2010). In accord with phytaspase cleavage specificity, an Asp residue is positioned at the prodomain–peptidase domain junction. This characteristic 'junction Asp' can serve as a phytaspase signature within the plant SBT family. Gain- and loss-of-function studies in *Nicotiana tabacum* confirmed the involvement of phytaspases in PCD. Overexpression of the phytaspase gene markedly enhanced PCD triggered by biotic (tobacco mosaic virus infection of *N* gene-containing plants) and abiotic (oxidative and high salt) stresses. Down-regulation of phytaspase activity in transgenic phytaspase-silenced plants and in wild-type plants treated with a phytaspase inhibitor, by contrast, suppressed PCD manifestations (Chichkova *et al.*, 2004, 2010). Thus, in the absence of aspartate-specific caspase orthologs in plants, subtilisin-like proteases appear to adopt, at least in part, their role in PCD.

Secretion of mature phytaspase into the apoplast under normal conditions of plant growth evidently protects intracellular proteins from phytaspase-mediated fragmentation. However, upon application of PCD-inducing stresses, phytaspases are rapidly relocalized back into the cell through a specific, as yet unknown mechanism, thus getting access to intracellular targets (Chichkova *et al.*, 2010; Fig. 4). Therefore, phytaspase-mediated cleavage of intracellular proteins is controlled not at the level of protease activity, as is the case for animal caspases, but rather by sequestration of the active enzyme in the apoplast and retrograde transport overcoming spatial separation from its intracellular substrates (Fig. 4; Vartapetian *et al.*, 2011; Chichkova *et al.*, 2012).

While cell death-related substrates are still unknown, a few phytaspase targets have been identified that are involved in biotic interactions. The VirD2 protein of phytopathogenic *Agrobacterium tumefaciens* is hydrolyzed by tobacco and rice phytaspases at a single site close to the C-terminus. This cleavage detaches the nuclear localization signal of VirD2 that is required for delivery of bacterial T-DNA into the plant cell nucleus. Phytaspase-mediated fragmentation of VirD2 was thus proposed to restrict transport of the T-DNA and integration into the nuclear genome (Chichkova *et al.*, 2004; Reavy *et al.*, 2007). A second phytaspase target, which is not originating from the pathogen but rather from the plant itself, is prosystemin, the precursor protein of the tomato wound hormone systemin (Beloshistov *et al.*, 2017). Prosystemin is cleaved by phytaspases purified from tomato leaves at two sites flanking the systemin sequence, thus excising the peptide hormone from its precursor. In agreement with this *in vitro* evidence for the involvement of phytaspases in prohormone processing, mutating the phytaspase cleavage sites in prosystemin abolished its defense
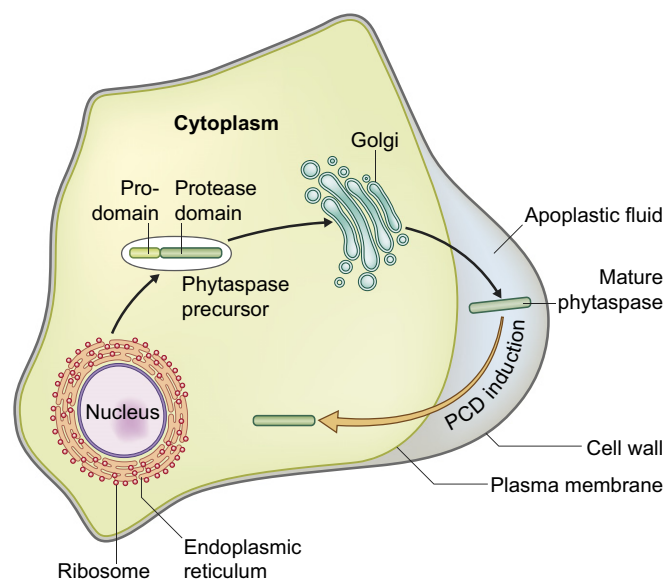
**Fig. 4** Control of phytaspase activity by sequestration in the apoplast. The phytaspase precursor is targeted to the secretory pathway and the mature and active enzyme is sequestered in the cell wall. Cell death-inducing insults trigger internalization of the active phytaspase, allowing access to intracellular substrates (modified from Chichkova *et al.*, 2012). PCD, programmed cell death.

signaling activity in tomato plants (Beloshistov *et al.*, 2017). Most recently, phytaspase was found to hydrolyze and inactivate the human gastrointestinal peptide hormones gastrin and cholecystokinin *in vitro* (Galiullina *et al.*, 2015). This finding prompted the idea to modulate concentrations of these human hormones in pathological conditions by ingestion of plant food with high phytaspase content.

Apart from phytaspases, two unidentified SBTs called saspases were isolated from oat (*Avena sativa*) treated with the death-inducing fungal toxin victorin (Coffeen & Wolpert, 2004). These enzymes displayed aspartate specificity of hydrolysis, although their preferred recognition motifs are different from those of phytaspases known from tobacco and rice. It is possible that saspases belong to the oat phytaspase group, yet a notable distinction of saspases is the increase in their activity in the apoplast upon death induction. Clearly distinct from phytaspases but also involved in cell death is the defense-related tomato SBT P69B. P69B is cleaved and inactivated by tomato matrix metalloproteinases (MMPs). P69B accumulates in cell walls of MMP-deficient tomato plants concomitant with spontaneous cell death that is initiated in epidermal and outer cortical cells of the hypocotyl (Zimmermann *et al.*, 2016). Cell death is suppressed upon silencing of P69B, suggesting that MMPs and SBTs act in an extracellular proteolytic cascade contributing to the regulation of cell death in tomato (Zimmermann *et al.*, 2016).

## 4. Plant SBTs as regulators of growth and development

Plant SBTs play important roles in shaping plant architecture, establishing the developmental fates of cells, and in cell-to-cell communication, thus modulating many different developmental

programs. They appear to be involved in virtually all stages of the plant life cycle, ranging from embryo and seed development (Tanaka *et al.*, 2001; D'Erfurth *et al.*, 2012) to germination (Rautengarten *et al.*, 2008), the mobilization of seed reserves (Qi *et al.*, 1992), vegetative growth (Srivastava *et al.*, 2008), reproductive development and senescence (Wang *et al.*, 2013; Martinez *et al.*, 2015; Schardon *et al.*, 2016). While knockout or overexpression mutants have provided solid evidence for the involvement of SBTs in many aspects of development, the exact mode of SBT action remains to be identified in most cases.

**Subtilases contributing to the regulation of cell wall structure** Several SBTs were found to affect cell wall structure and stability by regulating the activity of PMEs, and thus the methylesterification state of homogalacturonan as the major constituent of pectin. AtSBT6.1 (AtS1P) is required for the processing of the inhibitory prodomain and for export into the cell wall of group II PMEs (Wolf *et al.*, 2009). Likewise, AtSBT3.5 was shown to participate in PME processing during root development (Senechal *et al.*, 2014). AtSBT1.7 (ARA12), on the other hand, rather than processing PMEs, appears to negatively regulate PME activity during the later stages of seed development (Rautengarten *et al.*, 2008). In *AtSBT1.7* loss-of-function mutants, the apparent increase in PME activity results in de-methylesterification of seed mucilage. Consequently, mucilage fails to be released upon hydration, and germination of the *AtSBT1.7* mutant is reduced under low-water conditions (Rautengarten *et al.*, 2008). A similar phenotype was reported for PME Inhibitor 6 (PMEI6)-deficient plants, and the importance of AtSBT1.7 in addition to PMEI6 for down-regulation of PME activity and mucilage release was confirmed by the additive phenotype of the double mutant (Saez-Aguayo *et al.*, 2013).

**Subtilases contributing to root development, stem cell maintenance, and reproductive development** A role in root development was shown for AtSBT5.3 (Auxin-Induced in Root cultures 3, AIR3). AtSBT5.3 is expressed in the primary root, specifically in the outer cell layers at sites of lateral root formation, and its expression is enhanced by auxin (Neuteboom *et al.*, 1999). Whereas loss of *AtSBT5.3* function did not cause any phenotypic aberrations, constitutive overexpression leads to plants with longer lateral roots, without changes in lateral root density or cell wall abnormalities (Veth-Tello, 2005). AtSBT5.3 acts downstream of the transcription factor NAC1 mediating auxin-induced lateral root growth (Xie *et al.*, 2000). Lateral root growth is inhibited by high nitrate concentrations, and *AtSBT5.3* overexpression partially overcomes this inhibitory effect, suggesting that AtSBT5.3 activity is modulated by nutritional conditions (Veth-Tello, 2005).

Subtilases also seem to contribute to maintenance of the stem cell population at the shoot apical meristem, apparently by interacting with the CLAVATA3 (CLV3) signaling pathway. Ectopic overexpression of *AtSBT5.4*, a gene that is not normally expressed in the shoot apex, led to fasciated inflorescence stems, extrafloral organs and misshaped siliques resembling the *clv3* phenotype. Interestingly, the overexpression phenotype is more pronounced in *clv3* as compared with the wild-type background, resulting in the production of stem cell-like meristematic tissues and suppressing

organ formation (Liu *et al.*, 2009). While AtSBT5.4 appears to affect the CLV3 signaling pathway, it does not seem to be involved in the processing of the CLV3 precursor and the formation of the signaling peptide. An extracellular serine protease activity has been implicated in this process, but the enzyme has not been identified and it remains to be seen whether or not it belongs to the SBT family (Ni *et al.*, 2011).

Other SBTs are involved in reproductive development. The development of reproductive structures is modulated by AtSBT1.4 (Senescence Associated Subtilisin Protease, SASP; Martinez *et al.*, 2015). *AtSBT1.4* is expressed in all above-ground organs, particularly in senescing leaves, and its expression and activity are highly increased in plants at the reproductive stage. AtSBT1.4 down-regulates branching and silique production during monocarpic senescence, and its function is at least partially conserved between Arabidopsis and rice. A loss-of-function mutant (*sasp1*) exhibits more highly branched inflorescences, more siliques and increased seed yield. The increase in seed number is accompanied by a reduction in seed size. Seed size may be reduced in *sasp1* because of a decrease either in cell size or in cell number, as a result of competition for resources. In legumes, seed size depends on endosperm-localized SBT1.1 controlling cell number in cotyledons (D'Erfurth *et al.*, 2012). The apparent relevance of senescence-associated AtSBT1.4 for inflorescence branching (apical dominance), seed number and seed size suggests that the protease may act in a pathway for correlative control (influence of one organ over another), allowing seeds control over maternal resources and simultaneously restricting seed number (Wuest *et al.*, 2016).

**Subtilases contributing to the formation of signaling peptides** AtSBT2.4 was found to be involved in embryo development. Transposon tagging identified *AtSBT2.4* as the gene responsible for impaired epidermal functions and conditional lethality of the *abnormal leaf shape1* (*ale1*) mutant (Tanaka *et al.*, 2001). *AtSBT2.4* (*ALE1*) expression depends on ZHOUPI (ZOU), a transcription factor that also controls the expression of two leucine-rich repeat receptor-like kinases, GASSHO1 (GSO1) and GSO2, which act with AtSBT2.4 in the same genetic pathway regulating the formation of the embryonic cuticle in developing seeds (Tsuwamoto *et al.*, 2008; Yang *et al.*, 2008; Xing *et al.*, 2013). These findings suggest that AtSBT2.4 may be responsible for the formation of a peptide signal that activates the GSO1 and GSO2 receptor kinases. However, the substrate of AtSBT2.4 and the ligand of GSO1/GSO2 remain to be identified.

A role in the formation of an unidentified peptide signal was also proposed for stomatal density and distribution 1 (SDD1). The *SDD1* gene was identified in a forward genetic screen for mutations affecting stomata development in Arabidopsis. Loss of *SDD1* (*AtSBT1.2*) function results in a two- to fourfold increase in stomatal density (Berger & Altmann, 2000). Consistent with a role in stomata development, *AtSBT1.2* expression is weak in mesophyll cells, much higher in stomata initials and guard mother cells, but undetectable in mature guard cells (von Groll *et al.*, 2002). SDD1 negatively regulates the entry of protodermal cells into the stomatal lineage and the number of stomata produced per cell lineage. Therefore, stomatal density increases in the *sdd1* mutant and decreases in *SDD1*

overexpressor lines (Berger & Altmann, 2000; von Groll *et al.*, 2002). As stomatal density is of paramount importance for limiting water loss through the regulation of transpiration, either overexpression of *SDD1* or mutations of its transcriptional repressor GTL1 (GT-2 like 1) may reduce water loss and result in increased water-use efficiency (Yoo *et al.*, 2010; Liu *et al.*, 2015).

Suppression of stomata development by SDD1 depends on the leucine-rich repeat receptor-like protein Too Many Mouths (TMM; Nadeau & Sack, 2002; von Groll *et al.*, 2002), suggesting that SDD1 may be involved in the formation of a peptide signal as the ligand for TMM. While SDD1 targets have not been identified so far, Epidermal Patterning Factor 2 (EPF2), which also controls entry into the stomatal lineage by inhibiting protodermal cells from adopting the meristemoid mother cell fate (Hara *et al.*, 2009; Hunt & Gray, 2009), was found to be a substrate of AtSBT5.2 ($CO_2$ Response Secreted Protease, CRSP). Under high ambient $CO_2$, the EPF2 precursor is processed by AtSBT5.2 to restrict stomatal development (Engineer *et al.*, 2014).

The earlier examples implicate plant SBTs in the formation of peptides with hormone-like signaling properties. Signaling by small secreted peptides is, in fact, emerging as an important regulatory phenomenon in plant development (Matsubayashi, 2014; Tavormina *et al.*, 2015), and in some cases SBTs were shown to be involved. As mentioned earlier, the formation of the wound signal systemin involves phytaspases in tomato (Beloshistov *et al.*, 2017), while in Arabidopsis, AtSBT6.1 contributes to the formation of growth-regulating peptides, including RALFs and GOLVEN1 (Srivastava *et al.*, 2009; Ghorbani *et al.*, 2016; Stegmann *et al.*, 2017). However, the cleavage products generated by AtSBT6.1 from the respective precursor proteins are not the mature and bioactive peptides. Cleavage by AtSBT6.1 produces larger intermediates and, therefore, additional proteases are required for signal biogenesis. Similarly, AtSBT1.1 was implicated in the maturation of phytosulfokine (PSK), a sulfated pentapeptide serving as a noncell-autonomous signal for cell expansion, cell division in the root apical meristem, funicular pollen tube guidance, and the modulation of immune responses (Matsubayashi & Sakagami, 1996; Sauter, 2015). PSK is proteolytically processed from larger (80–120 amino acids) prepropolypeptides encoded by six different genes in Arabidopsis. One of the precursors, AtPSK4, is cleaved three amino acids upstream of the PSK sequence fairly specifically by SBT1.1 (Srivastava *et al.*, 2008). In SBT1.1 loss-of-function mutants, this cleavage does not occur (Srivastava *et al.*, 2008). However, despite the apparent requirement of SBT1.1 for AtPSK4 processing, no PSK-related phenotypes have been reported for the mutant. It is thus questionable whether or not processing by SBT1.1 is part of the endogenous PSK maturation process.

The biogenesis of Inflorescence Deficient in Abscission (IDA; Butenko *et al.*, 2003) and the abscission of floral organs, on the other hand, clearly depend on SBTs *in vivo*. When SBT activity is inhibited in abscission zones by tissue-specific expression of the Kazal inhibitors EPI1a or EPI10, floral organs fail to abscise (Schardon *et al.*, 2016). Redundant SBTs, including SBT4.13, SBT4.12 and SBT5.2, were found to be responsible for precursor

processing and release of the mature IDA peptide in abscission zones (Schardon *et al.*, 2016; Fig. 5). The released peptide binds to the extracellular leucine-rich repeat domain of the receptor-like kinases HAESA (Jinn *et al.*, 2000; Santiago *et al.*, 2016) and HAESA-LIKE2 (Cho *et al.*, 2008; Stenvik *et al.*, 2008), and promotes association with coreceptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family (Meng *et al.*, 2016; Santiago *et al.*, 2016). An intracellular MAP kinase cascade is activated (Cho *et al.*, 2008) and relays the signal to transcription factors regulating the expression of genes that execute the abscission process (Niederhuth *et al.*, 2013). Polygalacturonases and other hydrolytic enzymes degrade the pectin matrix in abscission zones, resulting in cell separation and the shedding of floral organs (Ogawa *et al.*, 2009; González-Carranza *et al.*, 2012; Niederhuth *et al.*, 2013). The IDA pathway is thus far the only peptide signaling pathway in plants, for which all elements have been identified, ranging from the peptide precursor to the processing proteases, the mature peptide signal, the receptors, intracellular signaling components, transcription factors and target genes.

We conclude that the function of (at least some) SBTs during plant growth and development resembles that of animal proprotein convertases. Several SBTs have been implicated in precursor processing for the formation of signaling peptides and, considering the size of the SBT family as well as the number of predicted peptide hormones, we anticipate that many more will be identified in the near future. In their role as proprotein convertases, SBTs exhibit redundancy and pleiotropy. They act redundantly, as several SBTs were found to process the same signals (e.g. processing of IDA by at least three SBTs; Schardon *et al.*, 2016). They have pleiotropic activities, in the sense that a single SBT may control different processes (e.g. SBT5.2 which is involved in organ abscission (Schardon *et al.*, 2016), stomatal density regulation (Engineer *et al.*, 2014), and plant defense (Serrano *et al.*, 2016)), depending on which SBT target proteins are expressed in the different tissues or developmental scenarios.

## V. Conclusions and outlook

Our progress in understanding the biochemistry and biological involvement of SBTs in plants has taken exciting and unexpected turns over the past 15 years. The widespread attempts to unravel the nature and function of plant SBTs, and their impact on a variety of signaling pathways, have led to exciting discoveries. SBTs were found to regulate plant development in most stages of the plant life cycle, from embryogenesis and organogenesis to senescence and PCD, and to play pivotal roles in many of the stress responses and adaptation mechanisms evolved by plants to their changing environment. Such multifaceted outputs of plant SBTs is, to some extent, reflected by the observation that SBTs are by far more numerous in plants than in other eukaryotes or prokaryotes. From the early discovery of cucumisin in melon fruits and of P69 in tomato plants in the 1990s, the more recent availability of genome sequences for many plant species has allowed researchers to expand the repertoire of SBTs and to begin to understand their evolution and functional diversification. On the other hand, it has become obvious that we have only scratched the surface with respect to SBT function and that there may be fundamental pathways linked to SBT activity that are yet to be discovered. Whatever these connections turn out to be, we are confident that exciting and important findings are still to come. Therefore, any advance in our understanding of SBT networks will help to reveal the basis and complexities of this remarkable signaling system in plants and provide a perspective on its origin and evolution. Moreover, although a few biologically relevant substrates have been identified, the difficulty of linking a given SBT to its physiological substrates represents a major bottleneck for further progress in understanding the molecular basis of how these proteases function. Likewise, we know very little of other proteins that may be required for SBT function and the regulation of SBT activity, and the molecular details of these potential interactions are not yet known. A sharper focus on these challenging areas of research will help to elucidate the
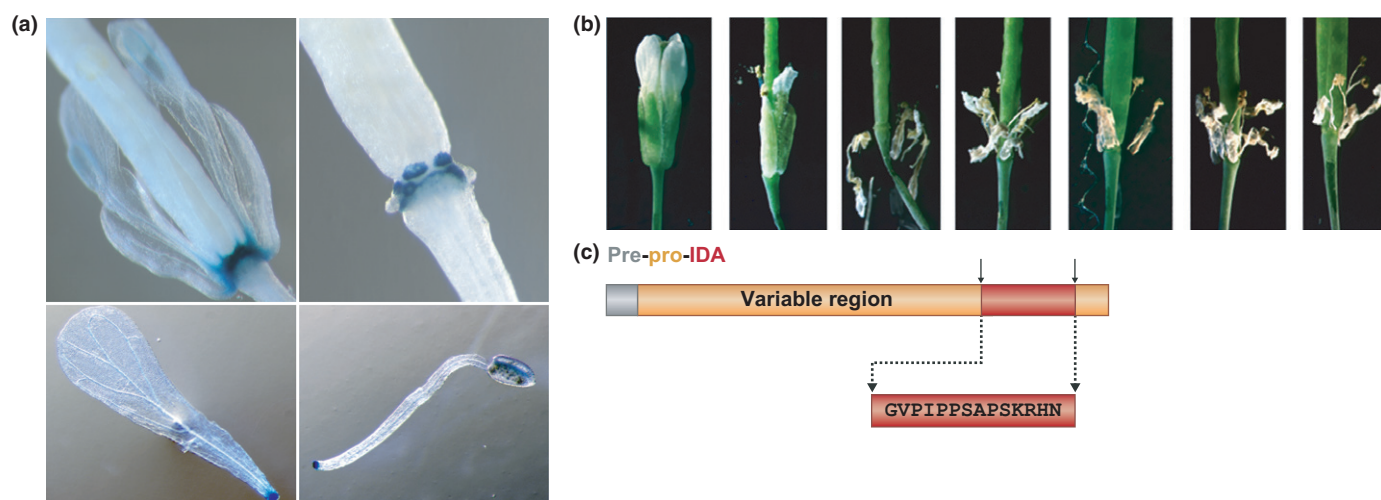


**Fig. 5** Subtilases (SBTs) in Inflorescence Deficient in Abscission (IDA) maturation and floral organ abscission. (a) Promoter:reporter (*uidA*) gene analysis indicates expression of SBTs in the abscission zones of floral organs in Arabidopsis flowers. (b) Floral organs do not abscise when SBT activity is inhibited in abscission zones by tissue-specific expression of the SBT-specific EPI10 inhibitor (Schardon *et al.*, 2016). (c) Precursor processing by several functionally redundant SBTs is required for the biogenesis of the mature IDA peptide (GVPIPPSAPSKRHN; Schardon *et al.*, 2016).

operational mode of SBTs and expand our vision of this proteolytic system regulating not only morphological transitions but also immune responses and stress adaptation in plants.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Phylogenetic relationship of Arabidopsis *SBT* genes.

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