1 INTRODUCTION

1.1 Wound Signaling in Plants

Large numbers of vertebrates and insects use terrestrial plants as food source. During their co-evolution over the last millions of years, plants have evolved several strategies to repel or hamper enemies that feed on them. Among these defense mechanisms, morphological adaptions, like thorns, trichomes, and toxic secondary plant metabolites, as well as a molecular defense have evolved. While morphological adaptations like thorns, or the incorporation of toxic secondary metabolites in the seeds and leaves mostly repel larger animals, theses defense mechanisms are often ineffective against adapted insect herbivores (Karban, Agrawal 2002; Wink, Theile 2002). Thorns can't repel insects, just because of their size, and many insects have evolved mechanisms to overcome the toxicity of secondary metabolites (Wink, Theile 2002; Mao et al. 2007). Beside the toxic secondary metabolites, defense related proteins are also involved in the direct defense against insect herbivores. These proteins target digestion enzymes in the insect's gut, degrade essential amino acids or disrupt the integrity of the insect's digestive tract (Felton et al. 1989; Pechan et al. 2002; Chen et al. 2005; Zhu-Salzman, Zeng 2015). Well-studied examples of such defense proteins are the protease inhibitors I and II (PI-I and –II), which inhibit digestive enzymes in the insect gut and prevent an efficient nutrient uptake (Vain et al. 1998; Urwin et al. 1997). Also, the well-studied polyphenol oxidases (PPOs) act in the defense against herbivorous insects by the formation of quinones which alkylate dietary proteins and degrade essential amino acids (Felton et al. 1989). Since the early investigations on proteinase inhibitors and polyphenol oxidases, in the 1980s and 90s, many other compounds have been identified, which contribute to plant defense against herbivores, like leucine aminopeptidase A, arginase, threonine deaminase, and many more (Staswick, Tiryaki 2004; Farmer et al. 1992; Fowler et al. 2009; Chen et al. 2005).

The central signaling pathway, which mediates the response to herbivorous insects, is the oxylipin pathway, which splits into the allene oxide synthase (AOS) and the hydroperoxide lyase (HPL) branches (Feussner, Wasternack 2002). Systemin, an 18 amino acid peptide in Solanaceae, which is released upon wounding from its precursor prosystemin by proteolytic cleavage, is the initial signal of this pathway and leads to

the formation of 13-hydroperoxy linolenic acid, the common substrate of the AOS- and HPL- branch (Turner et al. 2002; Pearce et al. 1991; McGurl et al. 1992; Matsui 2006). The outcome of the AOS branch is jasmonic acid (JA), which is transformed into its bioactive forms methyl jasmonate or JA-Ile, a conjugate of JA with isoleucine, mediating the downstream response of the plant to the herbivore threat (Farmer et al. 1992; Schaller 2008). The induction of defense genes in response to JA within hours is not only induced locally at the site of wounding, but also in unwounded, systemic tissues to enhance resistance against herbivore insects (Ryan 2000; Ryan, Pearce 1998). Another outcome of the oxylipin pathway are C_6-aldehydes, mainly n-hexenal, (Z)-3-hexenal and their derivates, the so called green leaf volatiles (GLVs) (Matsui 2006). GLVs are syntheized *de novo* upon insect attack and act as signal molecules eliciting the indirect defense by attracting natural enemies of the herbivore (Turlings et al. 1990; Kessler, Baldwin 2001; Pare, Tumlinson 1997). These chemical long distance signals are used to attract mainly parasitic wasps, which attack the herbivorous insects (Tumlinson et al. 1993).

1.2 Cell-Wall Modification by Pectin Methylesterases

Not only GLVs can act as signaling molecules in plant defense, but also methanol (MeOH), which is the second most abundant volatile compound in plants (Frenkel 1998; Guenther et al. 1995). MeOH signals affect the regulation of many defense related genes, but also genes related to developmental processes and stress responses (Downie 2004). Most of the MeOH produced in plants originates from the demethylesterification of pectin in the plant cell wall either during development or in response to herbivore attack (Dahl et al. 2006; Penuelas et al. 2005). Pectin methylesterases (PMEs), which mediate the demethlyesterification of pectin belong to large gene families. The genome of *Arabidopsis thaliana* codes for 66 PME genes and the one from *Populus trichocarpa* for 89, respectively (Pelloux et al. 2007). They mediate the demethylesterification of the homogalacturonane (HG) backbone of pectin, allowing cell wall modifying enzymes access to the pectin matrix. This includes hydrolases that target the pectin matrix, thereby loosening or even degrading the cell wall (Pelloux et al. 2007). Alternatively, demethylesterification also allows the formation of Ca^{2+} cross-links between different HG chains, increasing the rigidity of the cell wall (Pelloux et al. 2007).

PMEs are involved in many physiological processes like cell elongation, root morphology, shoot and pollen tube growth, and fruit ripening (Tieman, Handa 1994; Bosch et al. 2005; Derbyshire et al. 2007; Guenin et al. 2011). The overexpression of a fungal PME from *Aspergillus niger* in tobacco changed the entire cell wall metabolism and altered the morphology of the plant, leading to short internodes, small leaves, and a dwarf phenotype (Moustacas et al. 1991; Wen et al. 1999; Hasunuma et al. 2004). Another example is PME3 from Arabidopsis, which is involved in the formation of adventitious roots (Guenin et al. 2011). Beside their fundamental role in plant development, PMEs are also involved in biotic and abiotic stress responses. In winter oilseed rape (*Brassica napus*), for example, PME activity increased upon cold treatment and caused the formation of a stiff pectate gel, which is the result of an increased Ca^{2+}-crosslink between pectin fibrils (Solecka et al. 2008). The plant cell wall composition not only changes during cold stress, but also upon heat stress as shown in rice (*Oryza sativa*) seedlings. Here, a PME was induced after a heat shock and is supposed to grant other cell wall modifying enzymes access to the pectin gel, which in turn lead to cell wall loosening and liberation of Ca^{2+}. The increased apoplastic Ca^{2+} level is used in heat shock signaling and involved in the preservation of plasma membrane integrity (Wu, Jinn 2014). Increased PME activity during both cold and heat stress exemplifies the complexity of regulation within the plant cell wall, which is initiated by PME activity, but depends on the presence of other cell-wall modifying enzymes.

The composition and rigidity of the cell wall has also been shown to play a role in defense against pathogenic bacteria and fungi. The overexpression of a PME inhibitor (PMEI) in tobacco and Arabidopsis impaired the movement of tobamoviruses and reduced the susceptibility of the plants to that pathogen (Lionetti et al. 2014). Reduced activity of two PME isoforms also increased resistance to the necrotrophic fungus *Botrytis cinerea* in Arabidopsis (Lionetti et al. 2007). PMEs are also regulated upon herbivore attack, as seen for *Nicotiana attenuata* plants, which react with the upregulation of PME gene expression in response to feeding of *M. sexta* larvae, indicating an involvement of these enzymes in the defense reaction. (Giri et al. 2006). Hence, silencing of *Na*PME1 resulted in an increased performance of *Manduca sexta* larvae reared on these plants (Körner et al. 2009). This could either be caused by the toxicity of methanol, its function as a signaling molecule, or the modification of cell wall

properties (Liu 2003; Komarova et al. 2014; Dorokhov et al. 2012; Micheli 2001, 2001). Changes in the rigidity or composition of the plant cell wall could also contribute to the digestibility of the consumed leaves and possibly affect nutrient uptake in the insects digestive system.

Plant PMEs are divided into two groups, including group II PMEs that are synthesized as pro-proteins with an N-terminal propeptide, which shares similarities with a PME inhibitor (Markovic, Janecek 2004; Camardella et al. 2000; Pelloux et al. 2007). In order to become active, the inhibitor domain has to be processed, which occurs either in the late Golgi- apparatus or in the extracellular space (Wolf et al. 2009; Boudart et al. 2005; Mareck et al. 2012). In recent years, several proteases of the subtilase (SBT) family have been shown to be involved in the regulation of PMEs. In AtSBT6.1 (Site-1-protease, S1P) mutant plants, PME processing is impaired. The subtilase was found to interact with PMEs in co-immunoprecipitation experiments, showing a direct effect of AtSBT6.1 on PME maturation (Wolf et al. 2009). SBTs are not only involved in the activation, but also in the inactivation of PMEs by proteolytic cleavage. The subtilase SBT1.7 from Arabidopsis cleaves and thereby inactivates PMEs during the early stages of seed germination. In the SBT1.7 mutant, mucilage release after imbibition is prevented as a result of the increased PME activity (Rautengarten et al. 2008). In addition, AtPME17 and AtSBT3.5 are co-expressed in the root epidermis and in the root hair area. In co- infiltration experiments in *N. benthamiana*, specific cleavage of the PME pro-protein by SBT3.5 was observed, which resulted in the release of the active PME. PME17 and SBT3.5 knock out plants showed a similar phenotype with a decreased total PME activity and an increased primary root length (Sénéchal et al. 2014a).

These recent studies demonstrate how subtilase-mediated activation and inactivation of plant PMEs can affect cell wall properties in many different tissues and developmental stages.

1.3 Subtilases in Defense Responses

Also in wound signaling, our understanding of the regulation and fine-tuning of the different pathways is just beginning to emerge. While activation of pro-systemin by proteolytic cleavage is the initial step in wound signaling, the protease mediating this cleavage is still unknown. The same holds true also for the mechanisms of systemic

signal transduction into unwounded parts of the plant. In addition to electrical signals, systemin, oligogalacturonides and jasmonic acid have been proposed as long distance signals in the systemic wound response, but our knowledge how the signal is propagated and perceived in unwounded tissues is scarce (Schaller 2008; Howe, Schaller 2008). In all these individual steps, proteases are potentially involved and for many other defense pathways, proteases and specifically subtilases, have been shown to play an essential role.

The Arabidopsis subtilase SBT3.3, for example, is a key regulator in immune priming and induced resistance. The overexpression of SBT3.3 leads to enhanced resistance against pathogens like *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Ramírez et al. 2013). In cotton (*Gossypium barbadense*), the subtilase GbSBT1 recognizes a prohibitin (PHB)-like effector protein expressed by the fungus *Verticillium dahliae.* Kock-down of *GbSBT1* expression results in a loss of resistance to *V. dahlia* in *G. barbardense* plants and the stable transformation of Arabidopsis with *GbSBT1* resulted in resistance of the plants to *V*. *dahlia* and *Fusarium oxysporum.* The function of *Gb*SBT1 is directly linked to the oxylipin pathway, as *GbSBT1* expression was induced by jasmonic acid and the GbSBT1 protein re-localized from the cell membrane to the cytoplasm upon JA-treatment (Duan et al. 2016). Furthermore, the overexpression of *GbSBT1* in Arabidopsis plants led to an induction of JA-responsive genes, indicating that GbSBT1 can also induce the JA-signaling pathway (Duan et al. 2016).

1.4 Function and Maturation of Subtilases

Beside their role in defense mechanisms, more and more plant subtilases are also implicated in the regulation of stress responses and plant development. The involvement of subtilases in many of these processes is hardly surprising, as their homologues in mammals, the proprotein convertases (PCs), are involved in cell-cell communication, cholesterol biosynthesis and many diseases, like Alzheimer´s disease, inflammation reactions and tumor growth (Lan et al. 2010; Siegfried et al. 2003; Mumm et al. 2000; Kim et al. 2002). While only seven PCs are known in humans, the subtilase group hast drastically expanded in plants. With 56 members in Arabidopsis, 63 in rice and more than 90 SBT genes in P*opulus trichocarpa* (Tripathi, Sowdhamini 2006; Schaller et al. 2012; Rautengarten et al. 2005).

Their function ranges from unspecific protein degradation, which is known from catabolic prokaryotic subtilases, to the highly specific cleavage of precursor proteins as found in mammalian PCs. Cucumisin, which is found in the juice of melon fruits was the first characterized plant subtilase and is the type-example for degradative subtilases (Yamagata et al. 1994). On the other hand, our knowledge of SBTs with a high substrate specificity steadily increases. The Arabidopsis subtilases AtSBT1.1 and AtSBT6.1 (AtS1P) for example are required for the release of the active AtPSK4 and AtRALF23 peptides after specific cleavage of their prohormone precursors. The processed and thereby activated peptide hormones are involved in callus formation and hypocotyl elongation, respectively (Srivastava et al. 2008; Srivastava et al. 2009). The specific processing of peptide hormone precursors links plant subtilases to many developmental processes within a plants lifecycle. The subtilase SDD1 from Arabidopsis has been reported to cleave a yet unidentified substrate which is important in the regulation of the stomatal density on the leaf surface and contributes to the formation of stomatal patterning (Berger, Altmann 2000). Today, we know many examples, where plant peptides and proteins are processed at specific motives, whose sequences potentially match subtilase cleavage preferences. Unfortunately, we often do not know the exact subtilase, which is responsible for this cleavage. The identification of such subtilase-substrate pairs is a major task to investigate the role subtilases play in plants.

While little is known about the particular substrates of many plant subtilases, we have quite advanced knowledge of their biochemistry. The subtilase SBT3 from tomato, is the first plant subtilase that has been crystallized (Rose et al. 2009). SBT3 is synthesized, as most other subtilases, as a pre-pro-enzyme with an N-terminal signal peptide, which marks the nascent polypeptide for secretion. With entrance into the endoplasmatic reticulum (ER), the signal peptide is cleaved off and SBT3 undergoes maturation and post translational modification during its passage through the secretory pathway. This includes the formation of disulfide bonds, glycosylation and the cleavage of the propeptide (Cedzich et al. 2009; Ottmann et al. 2009). It was shown that propeptide cleavage is a prerequisite for secretion and that the processing is an intramolecular process. In active site mutants, where the catalytic serine residue at position 538 (Ser538) is mutated into an alanine (S538A) or cysteine (S538C), secretion was prevented and unprocessed SBT3 accumulated intracellularly. Also, the

co-expression of wild-type SBT3 with the active site mutants *in trans*, did not result in the secretion of the mutants into the apoplastic space, showing that zymogen maturation is indeed an autocatalytic and intramolecular process (Cedzich et al. 2009). While propeptide cleavage has been shown to be crucial for maturation and secretion of SBT3, our understanding of the propeptides function during enzyme maturation within the secretory pathway is limited.

1.5 Aims of the Present Work

The aim of this work was the characterization of the propeptide of tomato subtilase SBT3 during zymogen maturation with regard to typical features known from bacterial and mammalian propeptides. Furthermore, the function of mature SBT3 should be analyzed *in vivo*.

While an increasing number of plant subtilases has been characterized in recent years, we still have only rudimentary knowledge of the function of their propeptides. For bacterial and mammalian subtilases the propeptides have been described to have three distinct features. They act as chaperones, are strong inhibitors of the mature subtilase, and have to be cleaved in order to release the active subtilase.

A propeptide-deficient SBT3 mutant was generated and used to investigate the capability of its own and alien propeptides to act as chaperones during the maturation process of SBT3 in the secretory pathway. The interaction between the SBT3 mutant and different propeptides *in vivo* should give insights into the role of plant propeptides during zymogen folding and the specificity of this process. To characterize the interaction between SBT3 and different plant propeptides biochemically, SBT3 and different plant propeptides were purified and their inhibitory potential and their affinity towards mature SBT3 was measured.
The secondary structure of the SBT3 propeptide should be analyzed by CD-spectroscopy and by a structure-based alignment with other propeptides. Conserved or unique regions among them should be identified and compared with the ones in bacterial and mammalian propeptides to gain insights into functional relationships between them. Finally, the conditions leading to the release of mature SBT3 from the propeptide-inhibited complex and the underlying mode of action were analyzed and compared with that of other subtilases.

Additionally to the characterization of the propeptide, investigations on the biological function of mature SBT3 were made. Previous results indicated a possible role for SBT3 in the induced defense against insect herbivores.