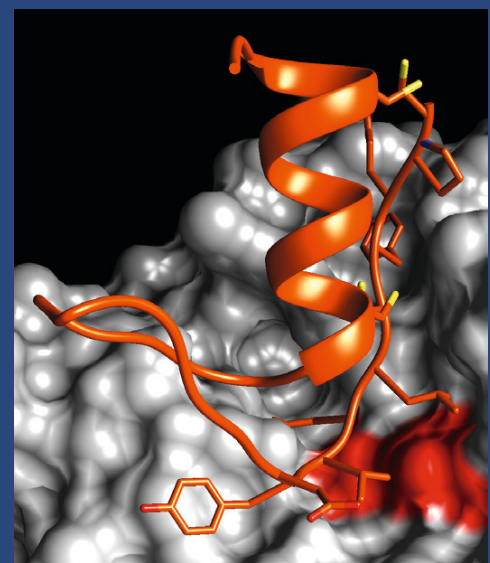
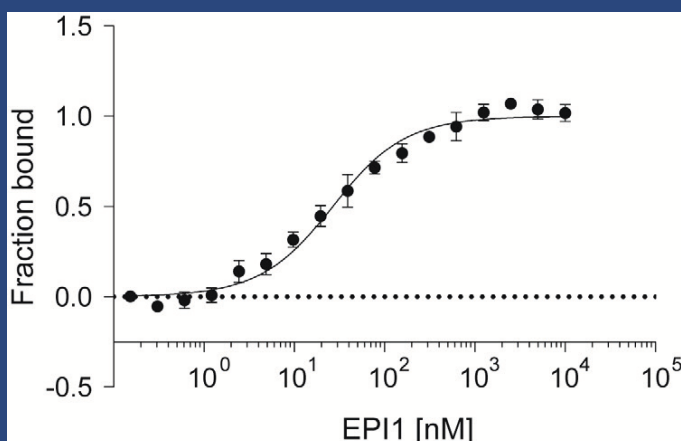
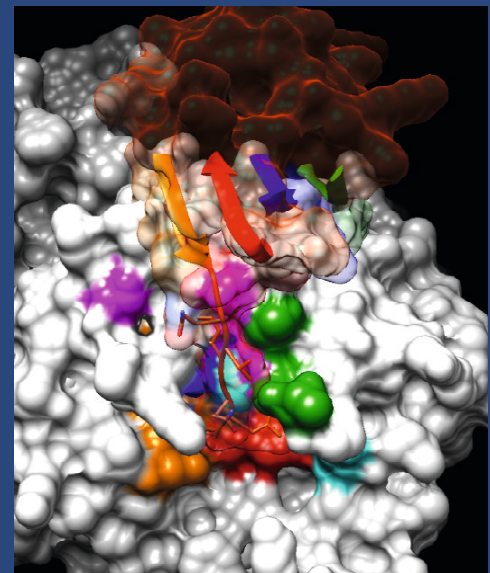
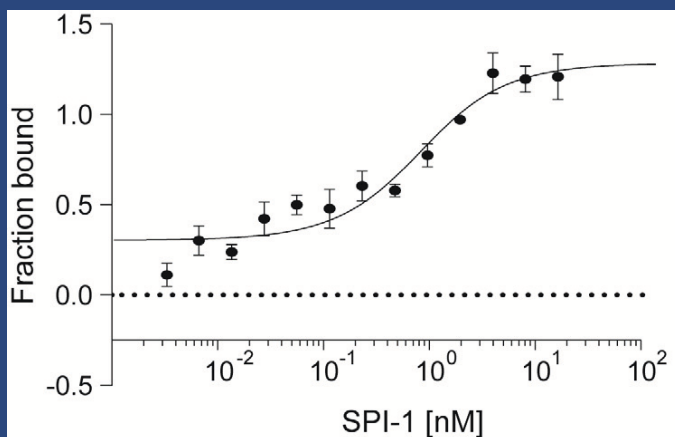


Mathias Hohl

Tissue-specific inactivation of subtilases in *Arabidopsis thaliana* by expression of proteinase inhibitors – a new approach to overcome functional redundancy

A. Schaller (Herausgeber) - Band 9





Schriftenreihe zur Physiologie und Biotechnologie der Pflanzen

Herausgeber: Prof. Dr. A. Schaller

Band 9/2017 Mathias Hohl

Tissue-specific inactivation of subtilases in
Arabidopsis thaliana by expression of proteinase
inhibitors
- a new approach to overcome functional
redundancy

D 100 (Diss. Universität Hohenheim)





Tissue-specific inactivation of subtilases in
Arabidopsis thaliana by expression of proteinase
inhibitors

- a new approach to overcome functional
redundancy

Dissertation zur Erlangung des Doktorgrades
der Naturwissenschaften (Dr. rer. nat.)

Fakultät Naturwissenschaften

Universität Hohenheim

Institut für Physiologie und Biotechnologie der Pflanzen

vorgelegt von

Mathias Hohl

aus Stuttgart

2017



Dekan: Prof. Dr. Heinz Breer

1. berichtende Person: Prof. Dr. Andreas Schaller

2. berichtende Person: Prof. Dr. Julia Fritz-Steuber

3. prüfende Person: Prof. Dr. Armin Huber

Eingereicht am: 02. 06. 2017

Mündliche Prüfung am: 13. 11. 2017

Die vorliegende Arbeit wurde am 18. 09. 2017 von der Fakultät Naturwissenschaften der Universität Hohenheim als "Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften" angenommen.

Bibliografische Information der Deutschen Nationalbibliothek

Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.d-nb.de> abrufbar.

1. Aufl. - Göttingen: Cuvillier, 2017
Zugl.: Hohenheim, Univ., Diss., 2017

© CUVILLIER VERLAG, Göttingen 2017

Nonnenstieg 8, 37075 Göttingen

Telefon: 0551-54724-0

Telefax: 0551-54724-21

www.cuvillier.de

Alle Rechte vorbehalten. Ohne ausdrückliche Genehmigung des Verlages ist es nicht gestattet, das Buch oder Teile daraus auf fotomechanischem Weg (Fotokopie, Mikrokopie) zu vervielfältigen.

1. Auflage, 2017

Gedruckt auf umweltfreundlichem, säurefreiem Papier aus nachhaltiger Forstwirtschaft.

ISBN 978-3-7369-9693-9

eISBN 978-3-7369-8693-0



CONTENT

LIST OF FIGURES.....	iv
SUMMARY.....	vii
ZUSAMMENFASSUNG.....	ix
1. INTRODUCTION	1
1.1 Regulation of proteolytic activity	1
1.2 Competitive inhibitors	3
1.3 Competitive inhibitors with exosite binding	4
1.4 Suicide inhibitors.....	6
1.5 Physiological roles of proteinase inhibitors in plants.....	7
1.6 Proteinase inhibitors in development and regulation of programmed cell death	8
1.7 Inter-species regulation of protease activity during defense	11
1.8 Subtilases.....	13
1.9 Plant peptide signaling and the involvement of SBTs in peptide processing.....	14
1.10 Aims of the present work	18
2. RESULTS.....	21
2.1 Precursor processing for plant peptide hormone maturation by subtilisin-like serine proteinases	21
2.1.1 <i>ABSTRACT</i>	22
2.1.2 <i>INTRODUCTION</i>	23
2.1.3 <i>RESULTS AND DISCUSSION</i>	24
2.1.4 <i>SUPPLEMENTAL FIGURES</i>	30
2.1.5 <i>EXPERIMENTAL PROCEDURES</i>	44
2.1.5.1 Generation of transgenic Arabidopsis expressing EP1a and EPI10 in abscission zone.....	44
2.1.5.2 Growth of experimental plants	45
2.1.5.3 Bioassay for abscission-inducing activity.....	45
2.1.5.4 Bioassay for PGAZAT-inducing activity	45
2.1.5.5 Cloning of SBTs and expression in <i>N. benthamiana</i>	46
2.1.5.6 Cleavage of IDA-GFP by co-expression with SBTs in <i>N. benthamiana</i>	49
2.1.5.7 Zymography of SBT activity and inhibition by EPI10.....	49
2.1.5.8 Expression and purification of EPI1a and EPI10	50



2.1.5.9	Purification of SBT4.12, SBT4.13 and SBT5.2.....	51
2.1.5.10	Expression and purification of GST-IDA	52
2.1.5.11	Processing of GST-IDA by SBT4.12, SBT4.13 and SBT5.2	53
2.1.5.12	Substrate specificity of SBT4.13 (PICS assay).....	53
2.1.5.13	Confirmation of substrate selectivity using Ala-substituted synthetic peptides	55
2.1.5.14	SBT4.12, SBT4.13 and SBT5.2 enzyme kinetics.....	56
2.1.5.15	Dissociation constants for EPI1a and EPI10 interaction with SBT4.13	57
2.1.5.16	Genetic complementation of <i>ida</i> with site-directed <i>IDA</i> and <i>ePIPP</i> mutants.....	57
2.1.5.17	Primers used for semi-quantitative RT-PCR	59
2.2	A novel subtilase inhibitor in plants shows structural and functional similarities to protease propeptides	61
2.2.1	<i>ABSTRACT</i>	62
2.2.2	<i>INTRODUCTION</i>	63
2.2.3	<i>RESULTS</i>	66
2.2.3.1	Phylogeny of I9 inhibitors and primary structure of SPI-1.....	66
2.2.3.2	Proteinase inhibitor activity and specificity of SPI-1	68
2.2.3.3	Kinetic analysis of SPI-1 binding and inhibition of SBT4.13	71
2.2.3.4	Characterization of the SPI-1/SBT4.13 complex.....	72
2.2.3.5	pH stability of the proteinase/inhibitor complex	75
2.2.3.6	Thermal stability of the proteinase/inhibitor complex.....	76
2.2.3.7	Assessment of SPI-1 as folding assistant.....	77
2.2.4	<i>DISCUSSION</i>	79
2.2.5	<i>EXPERIMENTAL PROCEDURES</i>	82
2.2.5.1	Cloning of SBTs	82
2.2.5.2	Cloning of the SBT4.13 propeptide deletion mutant, the PP of SBT4.13, and SPI-1 for expression in <i>N. benthamiana</i>	82
2.2.5.3	Transient protein expression in <i>N. benthamiana</i>	83
2.2.5.4	Purification of SBT4.13	83
2.2.5.5	Expression and purification of N-terminally His ₆ -tagged SPI-1	83
2.2.5.6	Protease activity against FITC-casein and inhibition by SPI-1	84
2.2.5.7	Inhibition of α -chymotrypsin and subtilisin A activity by recombinant SPI-1	84
2.2.5.8	SBT4.13 enzyme kinetics	85
2.2.5.9	Dissociation constants for the SPI-1/SBT4.13 complex.....	85
2.2.5.10	Determination of pH stability of the SBT4.13/SPI-1 complex.....	86



2.2.5.11	Analysis of the melting temperature of SBT4.13 and SBT4.13/SPI-1 inhibitor complex.....	87
2.2.5.12	Identification of the C-terminal cleavage site of SPI-1	87
3.	DISCUSSION.....	91
4.	BIBLIOGRAPHY.....	103
5.	APPENDIX	119
5.1	Primers for cloning of Prom::EPI constructs:	119
5.2	Modelling Methods	119
5.3	Model Building Report SBT4.13 (4yn3).....	121
5.4	Model Building Report SPI-1 (4yn3).....	124
5.5	Model Building Report EPI1 (4gi3).....	126
6.	DANKSAGUNG	129
7.	ERKLÄRUNG	131
8.	LEBENS LAUF	133



LIST OF FIGURES

Figure 1.1.	Schechter and Berger nomenclature of a protease/substrate complex.	3
Figure 1.2.	Competitive active site inhibition of proteases.	4
Figure 1.3.	Competitive inhibitors with exosite binding.	5
Figure 1.4.	Suicide inhibitors of endopeptidases.	7
Figure 2.1.1.	The shedding of floral organs depends on SBT activity in abscission zones.....	24
Figure 2.1.2.	mIDA restores abscission and IDA target gene expression in <i>IDAp::EPI10</i> transgenic lines.....	25
Figure 2.1.3.	Processing of the IDA precursor by SBTs is inhibited by EPIs.	26
Figure 2.1.4.	Identification of mIDA and processing site confirmation <i>in vivo</i>	28
Fig. S 2.1.1.	Subtilases expressed in abscission zones of the Arabidopsis flower.	30
Fig. S 2.1.2.	Promoter-reporter gene analysis of <i>SBT4.12</i> , <i>SBT4.13</i> and <i>SBT5.2</i> gene expression in Arabidopsis flowers.	31
Fig. S 2.1.3.	Inhibition of selected SBTs by recombinant EPI10.	31
Fig. S 2.1.4.	Expression and purification of the EPI inhibitors.	32
Fig. S 2.1.5.	Analysis of the <i>SBT4.12</i> , <i>SBT4.13</i> , and <i>SBT5.2</i> T-DNA insertion mutants....	33
Fig. S 2.1.6.	Analysis of IDA processing by selected abscission-zone SBTs.....	34
Fig. S 2.1.7.	Inhibition of <i>SBT4.13</i> by EPI1a and EPI10.	35
Fig. S 2.1.8.	Inhibition of <i>SBT4.12</i> by EPI1a and EPI10.	36
Fig. S 2.1.9.	Inhibition of <i>SBT5.2</i> by EPI1a and EPI10.	37
Fig. S 2.1.10.	Identification of mIDA as the processing product of GST-IDA.	39
Fig. S 2.1.11.	Purification and identification of <i>SBT4.13</i>	40
Fig. S 2.1.12.	Site-directed <i>IDA</i> mutants are expressed in abscission zones.	41
Fig. S 2.1.13.	Genetic complementation of <i>ida</i> with ePIPP and site-directed ePIPP mutants	42
Fig. S 2.1.14.	Residues relevant for cleavage site recognition by <i>SBT4.13</i>	42



Figure 2.2.1.	Phylogenetic relationship of propeptides and I9 inhibitors.	67
Figure 2.2.2.	Sequence alignment of SPI-1, homologues and SBT propeptides.	68
Figure 2.2.3.	Expression of Arabidopsis SBTs and inhibition by SPI-1.....	70
Figure 2.2.4.	Interaction of SBT4.13 with SPI-1.	71
Figure 2.2.5.	Purification of the SBT4.13/SPI-1 complex.	72
Figure 2.2.6.	Complex stability over time and C-terminal cleavage of SPI-1.	74
Figure 2.2.7.	pH stability and thermal stability of the SBT4.13/SPI-1 complex.	76
Figure 2.2.8.	Chaperoning activity of SBT4.13PP and SPI-1.....	78
Figure 3.1.	Alignment of EPI1 and EPI10 domains.	93
Figure 3.2.	Structural features of EPI1a and EPI1a complexed with subtilisin A.....	96
Figure 3.3.	Homology model SBT4.13/SPI-I complex based on the Cucumisin/PP complex	98

LIST OF TABLES

Table S 2.1.1:	Characterization of PICS assay peptide libraries.	43
Table 2.2.1:	Oligonucleotide primers used in this study (SPI-1).	89
Table A 5.3.1:	Template information for SBT4.13 homology model.....	121
Table A 5.3.2:	SBT4.13 model.....	123
Table A 5.4.1:	Template information for SPI-1 homology model	124
Table A 5.4.2:	SPI-1 model	125
Table A 5.5.1:	Template information for EPI1a homology model	126
Table A 5.5.2:	EPI1a model	127



ABBREVIATIONS

AvrBLB2	host-translocated RXLR-type effector protein	PGAZAT	Polygalacturonase Abscission Zone <i>Arabidopsis thaliana</i>
AvrRpt2	Cysteine protease avirulence protein 2	PI	peptidase inhibitor
C terminus	carboxy terminus	PICS	Proteomics Identification of Cleavage Sites
C14	cysteine protease 14	PIP1	Phytophthora-Inhibited Protease 1
CLV	Clavata	PIPP	IDA minimal motif required for receptor activation
DSF	differential scanning fluorometry	PMC	Potato Multicystatin
eIDA	extended IDA	PP	propeptide
EPF	Epidermal Patterning Factor	PR	pathogenesis-related
EPI	Kazal-like Extracellular Serine Protease Inhibitor	PRK5	Pollen-specific Receptor-like Kinase 5
EPIC	Extracellular Cysteine Protease Inhibitor	proIDA	precursor of IDA
ePIPP	extended PIPP	PSK	Phytosulfokine
ESI	electrospray ionisation	RALF	Rapid Alkalinisation Factor
FITC	fluorescein	RCL	reactive center loops
GLV	Golven	Rcr3,	Resistance cysteine protease 3
GST	Glutathione S-Transferase	RD21	Responsive-to-Desiccation-21
GUS	Glucuronidase	RIN4	RPM1-interacting protein 4
HAE	HAESA	ROS	reactive oxygen species
HSL2	HAESA-LIKE2	SAM	shoot apical meristem
IC ₅₀	concentration causing 50% inhibition	SBT	Subtilase
IDA	Inflorescence Deficient in Abscission	SD	standard deviation
K _d	dissociation constant	SDD1	Stomatal Density and Distribution 1
K _i	inhibition constant	SDS	sodium dodecyl sulfate
K _{iapp}	apparent K _i	SEM	standard error of mean
KO	knock-out	SERK	Somatic Embryogenesis Receptor Kinase
KTI	Kunitz's soybean Trypsin Inhibitor	serpin	Serine Protease Inhibitor
MALDI	matrix-assisted laser desorption ionization	SPI	Subtilase Propetide-like Inhibitor
MC	metacaspase	SRP	Serpin
mIDA	mature IDA	STM	Shoot Meristemless
MS	mass spectrometry	Subtilase	Subtilisin-like protease
MST	microscale thermophoresis	TIMP	human Tissue Inhibitors of Metalloprotease
N terminus	amino terminus	TOF	time of flight
nmIDA	IDA with N-methylated glycine	VPE	Vacuolar Processing Enzyme
OMTKY3	ovomucoid Kazal domain 3	wt	wild type
PAGE	polyacrylamide gel electrophoresis	XCP	Xylem Cysteine Protease
PC	prohormone convertase		

SUMMARY

The S8 family of subtilisin-like serine proteases (subtilases, SBTs), has largely expanded in plants, as seen for Arabidopsis, rice and potato, with 56, 63, and 80 members, respectively. Like mammalian proprotein convertases, plant subtilases are involved in the activation of proproteins by limited proteolysis. Due to functional redundancy, most single-gene loss-of-function mutants lack obvious phenotypes. Functional redundancy was addressed here in a biochemical approach using SBT-specific inhibitors to impair SBT function at the level of enzyme activity rather than gene expression.

Extracellular Proteinase Inhibitors (EPIs), from the oomycete plant pathogen *Phytophthora infestans* were tissue-specifically expressed under the control of promoters from different peptide hormone genes. Transgenic Arabidopsis plants expressing EPIs under control of the *IDA* (*Inflorescence Deficient in Abscission*) promoter showed the *ida*-characteristic defect in floral organ abscission. The abscission defect was complemented by external application of the mature IDA peptide, whereas the application of processable and unprocessable precursor peptides showed a reduced and no effect, respectively. Arabidopsis SBT4.12, SBT4.13 and SBT5.2 were found to cleave the IDA precursor to release the mature IDA peptide. All three proteases were potently inhibited by EPIs *in vitro*. The cleavage preference of SBT4.13 reflected the IDA processing site, while alanine substitutions for proline and tyrosine, two and four amino acids upstream of the IDA processing site, strongly reduced cleavage efficiency by SBT4.13 *in vitro*. Expression in the *ida* mutant background revealed reduced complementation efficiency for the modified precursor peptides as compared to the wild type, indicating that SBT-mediated cleavage at this site is required *in vivo* for the biogenesis of the IDA signalling peptide.

Furthermore, a SBT Propeptide-like Inhibitor (SPI-1) was identified in Arabidopsis and characterized with respect to its inhibitory potential and suitability for the inhibitor-based loss-of-function approach. Phylogenetic analysis identified SPI-1 homologues in all land plants including mosses, and a distant relationship to subtilase propeptides. The chaperoning activity typically found in SBT propeptides was not observed for SPI-1. The inhibitor profile of SPI-1 included many Arabidopsis subtilases, while bacterial subtilisin A or bovine chymotrypsin were not inhibited. SPI-1 showed 20 to 40-fold higher affinity to SBT4.13 than EPIs, and the SBT4.13/SPI-1 complex was highly stable at a broad pH range from pH5 to 10. These results

indicate that SPI-1 may be a good alternative to EPIs in attempts to control subtilase activity *in vivo*.

Using proteinase inhibitors to impair protease function at the level of enzyme activity is a promising approach, if classical genetic methods are not feasible due to genetic and functional redundancy. However it is important to note, that the choice of protease inhibitor is crucial for the success of this approach. Recommended are “standard mechanism” serine protease inhibitors, propeptide-like I9 inhibitors or serpins, which bind their targets in a substrate-like manner. The active sites of these inhibitors can be modified to match the substrate specificity of the targeted proteases reflecting the cleavage-site sequence of substrates of interest. The proposed experimental approach is not limited to proteases. Whenever peptide-based enzyme inhibitors exist for families of functionally redundant enzymes, they can be used for loss-of-function analysis by targeting enzyme activity rather than gene expression.

ZUSAMMENFASSUNG

Subtilasen gehören zur S8 Familie der Subtilisin-ähnlichen Serinproteasen (Subtilasen, SBTs). Diese Proteinfamilie ist in Pflanzen stark expandiert und besitzt 56, 63 und 80 Angehörige in Arabidopsis, Reis und Kartoffel. Pflanzliche Subtilasen sind, wie ihr tierisches Pendant, die Propeptidkonvertasen, durch limitierte Proteolyse an der Aktivierung von Proteinvorläufern beteiligt. Auf Grund von funktioneller Redundanz zeigen jedoch die meisten T-DNA-Insertionslinien einzelner Subtilasegene keinen Phänotyp. Diese funktionelle Redundanz wurde in einem biochemischen Ansatz mit SBT-spezifischen Inhibitoren überwunden, indem die Funktion von SBTs auf Enzym- und nicht wie sonst üblich auf Expressionsebene reguliert wurde.

Extrazelluläre Proteinaseinhibitoren (EPIs), des pflanzenpathogenen Oomyceten *Phytophthora infestans*, wurden gewebespezifisch unter Kontrolle von Promotoren verschiedener Peptidhormone exprimiert. Transgene Arabidopsispflanzen, die EPIs unter Kontrolle des *IDA* (*Inflorescence Deficient in Abscission*) Promotors exprimierten, wiesen den *ida*-charakteristischen Defekt in der Abszission von Blütenorganen auf. Dieser Abszissionsdefekt konnte durch die externe Applikation des reifen IDA Peptides komplementiert werden, wohingegen die Applikation eines spaltbaren und eines nicht spaltbaren Vorläufers einen verminderten, bzw. keinen Effekt aufwies. Arabidopsis SBT4.12, SBT4.13 und SBT5.2 waren in der Lage das reife IDA Peptid, zu generieren, während deren Aktivität *in vitro* durch EPIs vollständig blockiert wurde. Die Spaltpräferenz von SBT4.13 spiegelte sich, in der Peptidsequenz der IDA Spaltstelle wieder. Ein Austausch von Prolin und Tyrosin zu Alanin zwei bzw. vier Aminosäuren oberhalb der Spaltstelle hatten einen deutlich negativen Effekt auf die Spalteffizienz durch SBT4.13 *in vitro* und eine Expression dieser Peptidmutanten im *ida* Hintergrund zeigte ein deutlich reduziertes Potential die *ida* Mutante zu komplementieren. Dies weist darauf hin, dass SBTs für die aktivierende Spaltung des IDA Vorläufers essentiell sind.

Des Weiteren wurde ein Subtilase Propeptide-ähnlicher Inhibitor (SPI-1) identifiziert und bzgl. seines Inhibitorpotentials und seiner Eignung für den inhibitorbasierten Ansatz SBT Aktivität zu regulieren, funktionell charakterisiert. Phylogenetische Analysen von SPI-1 zeigten homologe Inhibitoren in allen Landpflanzen, inklusive in Mosen, aber nur entfernte Verwandtschaft zu SBT Propeptiden. Die für SBT Propeptide charakteristische Chaperonfunktion konnte für SPI-1 nicht beobachtet werden. SPI-1 zeigte eine breite Hemmungswirkung von Arabidopsis SBTs, während weder bakterielles Subtilisin A noch

bovines Chymotrypsin gehemmt wurden. Die Bindungsaffinität von SPI-1 zu SBT4.13 war 20 bis 40-fach höher als die von EPI Inhibitoren und der SBT4.13/SPI-1 Komplex war über einen weiten pH Bereich von pH5 bis pH10 stabil. Diese Ergebnisse deuten darauf hin, dass SPI-1 eine interessante Alternative zu EPI Inhibitoren, für die Regulation von Subtilasen *in vivo* sein könnte.

Die Nutzung von Proteaseinhibitoren zur Regulation von Proteaseaktivität auf Enzyme Ebene ist ein vielversprechender Ansatz, wenn klassische genetische Ansätze auf Grund von funktioneller oder genetischer Redundanz nicht einsetzbar sind. Ausschlaggebend für den Erfolg des Ansatzes ist dabei die Auswahl des richtigen Proteaseinhibitors. Deshalb wird die Verwendung von „Standard Mechanismus“ Serinproteaseinhibitoren, propeptid-ähnlichen I9 Inhibitoren oder Serpinen empfohlen, da diese von der Zielprotease ähnlich gebunden werden wie ein Substrat. Diese Inhibitoren können in ihrem aktiven Zentrum durch den Austausch der Bindedomäne gegen die Spaltstelle eines zu analysierenden Substrates modifiziert werden, um der Substratspezifität von Zielproteasen zu entsprechen. Dieser inhibitorbasierte Ansatz ist nicht limitiert auf Proteasen. Sofern Peptid-basierte Enzyminhibitoren bekannt sind lässt sich dieser Ansatz entsprechend auch auf andere funktionell redundante Enzymgruppen anwenden.



1. INTRODUCTION

Functional redundancy describes the situation when two or more genes are responsible for the same biochemical function, resulting in no or minor biological effects if the activity of one gene is lost. In most cases redundancy is a result of gene duplications and it is a widespread phenomenon in higher organisms (Nowak *et al.*, 1997). While it improves the robustness of essential biological reactions it is a major drawback during genetic analysis of knock-out (KO) mutants, as single gene KOs will show no phenotypic alterations. In order to assess this, the current project focussed on a biochemical approach to control functional redundancy of proteolytic enzymes on the enzyme level. To establish such an approach it is necessary to understand how proteolytic activity is regulated.

1.1 Regulation of proteolytic activity

Proteolytic enzymes deal with a plethora of tasks, ranging from general protein degradation to very specific regulatory processes including the activation of zymogens, cleavage of signal peptides or the activation of peptide hormones. These processes are involved in the regulation of various developmental processes, as well as cell death and responses to wounding or pathogenic threats. Peptidases are found in all organisms, constituting 2-4% of all encoded gene products (Farady & Craik, 2010), and they are believed to have evolved over time from general protein degrading enzymes to regulators of increasing specificity (Neurath, 1984). Peptidases cleaving at internal and terminal cleavage sites are called endopeptidases and exopeptidases, respectively. The latter are subdivided in carboxy- and aminopeptidases. Peptidases are further separated based on their catalytic mechanisms into the six different classes of serine, cysteine, glutamic, threonine, aspartate and metallo-proteases. The major difference between these classes is the nature of their catalytic residues, which are involved in the nucleophilic attack on the substrate peptide bond. While serine, cysteine and threonine proteases use Ser, Cys and Thr as nucleophile (Polgár, 2013a,b; Rawlings & Barrett, 2013), metallo-, aspartic and glutamic proteases use water as a nucleophile (Auld, 2013; Wlodawer *et al.*, 2013), and are activated by a metal ion or Asp and Glu, respectively. While the human genome codes for 612 proteases, in *Arabidopsis* an even higher number of 826 proteases is predicted, including serine proteases as their largest class (Van Der Hoorn, 2008; Farady & Craik, 2010). Considering this high variety of proteases and the fact that the process of proteolysis is essentially irreversible, not only their