SNARE-mediated plant immune responses at the cell periphery

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Table of contents

Abstract

Pre-invasion resistance responses of *Arabidopsis* to the non-adapted barley powdery mildew fungus *Blumeria graminis* fsp *hordei* (*B. g. hordei*) require at least four *PEN* (*penetration*) genes. *PEN1* to *PEN4* encode a syntaxin, a ß-glycosyl hydrolase, an ABC transporter, and a γ-glutamylcysteine synthetase, respectively. Epistasis analysis suggests that the PEN1 syntaxin acts in a pathway that is different from a second pathway comprising PEN2, PEN3, and PEN4. Syntaxins are members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein super family mediating intracellular vesicle trafficking processes in eukaryotic cells. In animals and yeast, syntaxins direct vesicle trafficking by forming ternary SNARE complexes with a SNAP25 adapter protein and a vesicle-resident v-SNARE (VAMP).The isolation of four independent *pen1* alleles, each supporting enhanced cellular entry of *B. g. hordei* condidiospores, provided for the first time genetic evidence for the possible existence of a vesicle-based and secretory disease resistance mechanism at the cell periphery mediated by a single syntaxin family member.

My work aimed to investigate PEN1 structure-function relationships using transgenic *Arabidopsis* plants that express engineered PEN1 variants at native levels in a *pen1*-1 null mutant background. Single amino acid substitutions that have previously been reported to affect the activity of syntaxins in *Rattus norvegicus*, *Caenorhabditis elegans*, and *Drosophila melanogaster* were introduced into the PEN1 sequence to generate a first set of PEN1 variants. Functional analysis of the respective Arabidopsis transgenic lines revealed that amino acid residues in and adjacent to the conserved SNARE domain are required for full PEN1 activity in disease resistance to *B. g. hordei*, thereby supporting the idea that PEN1 functions in this biological process like an authentic syntaxin that involve SNARE-SNARE domain interactions.

Additional PEN1 variants involved N-terminal serine substitutions that were previously found to be phosphorylated in cultured *Arabidopsis* cells upon elicitation with the bacterial-derived flg22 peptide, which is recognized by the plasma membrane-resident FLS2 immune receptor. Phosphorylation of N-terminal residues upon flg22 elicitation has also been reported in the closely related family member, syntaxin SYP122. Transgenic lines expressing PEN1 phospho-mimic variants show wild-type-like PEN1

activity, but elevated *B. g. hordei* entry rates of lines expressing phospho-knockout derivatives suggest that N-terminal phosphorylation events modulate PEN1 activity during disease resistance responses.

Unlike PEN1, a marked pathogen-inducible increase in protein levels of SYP122 was found only at late time points upon *B. g. hordei* challenge, raising the possibility that the apparent functional diversification of the closely related family members might be due to their differential accumulation patterns. However, constitutive overexpression of SYP122 could not complement the *pen1* mutant phenotype although PEN1 overexpressing lines restored disease resistance to *B. g. hordei*. This suggests that in disease resistance to *B. g. hordei* the functional diversification between PEN1 and SYP122 is complete.

Functional GFP-tagged PEN1 has previously been shown to accumulate beneath attempted powdery mildew entry sites. I found that the candidate interacting SNARE proteins SNAP33 and VAMP722 co-localized with PEN1 at such sites. Interestingly, non-functional PEN1 variants also accumulate at fungal entry sites, indicating that the focal accumulation is not a marker of PEN1 activity. I discuss a model in which PEN1 accumulation at fungal entry sites and PEN1 activity in disease resistance are separate biological processes.

Zusammenfassung

An prä-invasiven Abwehrmechanismen von Arabidopsis gegen den Gerstemehltau Blumeria graminis fsp hordei sind mindestens vier PEN-Gene ("Penetrationsgene") beteiligt. Diese Gene (PEN1 bis PEN4) kodieren für ein Syntaxin, eine ß-Glycosyl-hydrolase, einen ABC-Transporter und eine γ-Glutamylcysteinsynthetase. Durch Analyse der genetischen Interaktionen der PEN-Gene konnte eine Funktion für das PEN1 Syntaxin in einem von PEN2, PEN3 und PEN4 unabhängigen zellulären Mechanismus gezeigt werden. Syntaxine gehören zur Protein-Superfamilie der SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), die an intrazellulären Vesikeltransportprozessen in eukaryotischen Zellen beteiligt sind. Es konnten vier unabhängige Allele von pen1 isoliert werden, die alle eine erhöhte Eintrittsrate des Gerstenmehltaus B. g. hordei aufwiesen. Diese Syntaxin Defektallele lieferten zum ersten Mal genetische Hinweise auf einen möglichen Vesikel-basierten sekretorischen Resistenzmechanismus an der Peripherie der Zelle, der durch ein einzelnes Mitglied der Syntaxin-Familie getragen wird. Aus Tieren und Hefen ist bekannt, dass Syntaxine durch Komplexbildung mit SNAP25 Adaptorproteinen und Vesikel-assozierten VAMP Proteinen (vesicle associated membrane protein) an der Vermittlung von Vesikelfusionprozessen an Zielmembranen beteiligt sind.

Ziel dieser Arbeit war es, eine Struktur-Funktionsanalyse von PEN1 in transgenen Arabidopsis-Pflanzen durchzuführen, die verschiedenen Varianten von PEN1 in nativen Mengen in einem Nullmutanten-Hintergrund exprimieren. Durch Funktionsanalyse von transgenen Arabidopsis-Pflanzen, die PEN1-Varianten mit einzelnen Aminosäure-austauschen exprimieren, für die bekannt ist, dass sie die Aktivität von Syntaxinen in Rattus norvegicus, Caenorhabditis elegans und Drosophila melanogaster beeinflussen, konnte in der vorliegenden Arbeit gezeigt werden, dass Aminosäuren innerhalb und nahe der konservierten SNARE-Domäne für volle Aktivität von PEN1 in der Resistenzreaktion gegen B. g. hordei nötig sind. Dieser Befund unterstützt die Hypothese, dass PEN1 in diesem biologischen Prozess als authentisches Syntaxin über SNARE-Domänen-Interaktionen fungiert. Weitere generierte und analysierte PEN1-Varianten, beinhalteten Serin-Substitutionen am Aminoterminus von PEN1. Für die hier ausgetauschten Serinreste ist in Arabidopsis-Zellkulturen eine Phosphorylierung nach Induktion mit dem bakteriellen flg22-Peptid, das durch den Plasmamembranrezeptor

FSL2 erkannt wird, gezeigt worden. Eine ähnliche flg22-induzierte Phosphorylierung an aminoterminalen Serinresten ist auch für das sequenzverwandte SYP122 Syntaxin von Arabidopsis gezeigt worden. Interessanterweise zeigten transgene Pflanzenlinien, die Phosphorylierungsimitationsvarianten von PEN1 exprimierten, Wildtyp-ähnliche Pilzeintrittsraten. Hingegen zeigten transgene Pflanzenlinien mit unphophorylierbaren PEN1-Varianten erhöhte Eintrittsraten des Gerstemehltaus. Dieser Befund weist darauf hin, dass N-terminale Phosphorylierungereignisse die Aktivität von PEN1 in Abwehrreaktionen modulieren.

Im Vergleich zu PEN1 zeigt SYP122 einen deutlichen pathogen-induzierten Anstieg in der Proteinmenge zu späten Zeitpunkten nach Inokulation mit Gerstenmehltau. Die scheinbare funktionelle Diversifizierung könnte dementsprechend zwischen beiden verwandten Proteinen auf ihren unterschiedlichen Proteinmengen beruhen. Allerdings konnte eine Überexpression von SYP122 im pen1 Nullmutanten-Hintergrund die erhöhte pilzliche Eintrittsrate des pen1 Phänotypen nicht komplementieren, wohingegen eine Überexpression von PEN1 die Resistenz gegen B. g. hordei Eintritt wiederherstellen konnte. Das weist daraufhin, dass die funktionelle Diversifizierung zwischen PEN1 und SYP122 in der Eintrittsresistenz gegen Gerstenmehltau vollständig ist.

Es ist bekannt, dass funktionales GFP-markiertes PEN1 unter versuchten Eintrittsstellen des Gerstenmehltaus akkumuliert. Hier konnte gezeigt werden, dass die potenziellen SNARE-Interaktionspartner von PEN1, SNAP33 und VAMP722, mit PEN1 an solchen Stellen ko-lokalisieren. Interessanterweise akkumulieren auch nicht-funktionale PEN1 Varianten an dieser Stelle, was zeigt, dass die fokale Akkumulierung kein Marker für PEN1 Aktivität ist. Ich schlage ein Modell vor, in dem PEN1 Akkumulierung und PEN1 Aktivität zwei unterschiedliche Prozesse darstellen.

Abbreviations

% (v/v)	volume percent
% (w/v)	weight/volume percent
3’	downstream region (of a gene or sequence)
5’	upstream region (of a gene or sequence)
µ	micro
A	alanine
aa	amino acid
ala	alanine
ATP	adenosine trisphosphate
Avr	avirulence gene
At, A. thaliana	*Arabidopsis thaliana*
Arabidopsis	*Arabidopsis thaliana*
B. g. hordei	*Blumeria graminis* fsp *hordei*
bla	ß-lactamase
°C	degrees Celsius
Ca^{2+}	calcium ions
CAPS	cleaved amplified polymorphic sequence
cDNA	copy DNA
Ce, C. elegans	*Caenorhabditis elegans*
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
C-terminus	carboxy terminus
CUb	C-terminal half of ubiquitin
D	aspartate
dH_2O	de-ionized water
Dm	*Drosophila melanogaster*
DMSO	dimethyl sulfoxide
DNA	desoxy ribonucleic acid
dNTPs	desoxyribonucleotides
DTT	dithiothreitol
E	Euler number
E	glutamate

E. cichoracearum	*Erysiphe cichoracearum*
ECL	enhanced chemi-luminescence
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetate
EMS	ethyl methane sulfonate, or methane sulfonic acid ethyl ester
ER	endoplasmic reticulum
ET	ethylene
FOA	5' fluoroorotic acid
FRET	Förster resonance energy transfer
fsp	forma specialis
g	gram
G	glycine
GFP	green fluorescent protein
GTP	guanidine trisphosphate
GW	gateway
h(rs)	hour(s)
H	histidine
HA	hemagglutinin
his	histidine
hpi	hours post inoculation
HR	hypersensitive response
HRP	horse radish peroxidase
Hv	*Hordeum vulgare*
I	isoleucine
i.e.	id est
JA	jasmonic acid
K	kilo
Kb	kilo base
kD	kilo Dalton
l	liter
L	leucine
Le	*Lycopersicon esculentum*
leu	leucine
lys	lysine

m	milli
M	molar (mol/l)
min	minutes
MLO	mildew resistance locus o
mRNA	messenger RNA
Mt	*Medicago truncatula*
mYFP	monomeric yellow fluorescent protein fluorescent protein
n	nano
NASC	Nottingham *Arabidopsis* Stock Center
Nb	*Nicotiana benthamiana*
nm	nano meter
NMR	nuclear magnetic resonance
NSF	*N*-ethylmaleimide sensitive factor
Nt	*Nicotiana tabacum*
N-terminus	amino terminus
NuI	N-terminus of ubiquitin I
OD	optical density
P	probability value
p35S	promoter of Cauliflower mosaic virus promoter 35S
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PAT	phosphinothricin-acetyltransferase
PCR	polymerase chain reaction
pH	negative logarithm of proton concentration
PR	PAMP recognition receptor
PBS	phosphate buffered saline
PEN1	penetration 1
Pfu	*Pyrococcus furiosus*
PM	plasma membrane
pPEN1	promoter of PEN1
RLK	receptor like kinase
Rn, R. norvegicus	*Rattus norvegicus*
RNA	ribonucleic acid
ROR2	required for *mlo* base resistance

rpm	rounds per minute
s	seconds
S	serine
SA	salicylic acid
SAG101	*senescence associated gene 101*
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
α-SNAP	soluble NSF attachment protein
SNAP25	synaptosomal protein of 25kD
SNAP33	synaptosomal protein of 33kD
SNARE	soluble NSF attachment protein receptor
SM	Sec/Munc
SOE	splice site overlap extension
SYP	syntaxin of plants
T	tryptophane
T_1	first filial generation after transformation
T_2	second filial generation after transformation
T_3	third filial generation after transformation
Taq	*Thermophilus aquaticus*
TBS	tris buffered saline
TBS-T	TBS with 0,5% Tween-20
TLR	Toll like receptor
trp	tryptophane
t-test	statistical hypothesis test by Student (1908)
u	(enzymatic) unit
U	uracile
ura	uracile
URA3	orotidine-5´-phosphate decarboxylase
V	valine
V	volt
v	volume
w	weight
w/o	without
wt	wild type

I Introduction

I.1 SNARE protein family members mediate vesicle fusion at target membranes

Intracellular vesicle trafficking and targeted membrane fusion is a key feature of eukaryotic cells. This process constitutes cargo transport in membrane-enclosed vesicles between organelles and the surrounding of the cell (Chen and Scheller 2001, Jahn and Grubmuller 2002, Jahn et al. 2003, Sanderfoot et al. 2000). By now, almost three decades of research have highlighted the importance of members of the SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) protein family in facilitating such intracellular membrane fusion events (Schekman and Novick 2004). SNARE protein family members are characterized by the presence of a coiled-coil heptad repeat motif (designated the SNARE domain) and often contain additional membrane spanning helices (Jahn and Grubmuller 2002, Jahn et al. 2003, Sutton et al. 1998). Current knowledge from numerous studies in yeast and animal systems, as well as the presence of SNAREs in all eukaryotic genomes suggests a conserved mechanism of membrane fusion across kingdoms (Bock et al. 2001, Ferro-Novick and Jahn 1994, Sanderfoot 2007). Consequently, SNARE proteins have been hypothesized to participate in all intracellular membrane fusion events in eukaryotic cells (Bock et al. 2001, Chen and Scheller 2001, Hong 2005).

SNAREs have been defined as Qa, Qb, Qc and R-types according to the central amino acid in the SNARE domain (Bock et al. 2001, Fasshauer et al. 1998). It is thought that SNARE-mediated membrane fusion events require the formation of a highly stable trans-membrane SNARE complex, the so-called ternary SNARE complex (Weber et al. 1998, Weninger et al. 2003). The core complex has been proposed to contain one member of each of the four types of SNARE domains (Qa Qb Qc R rule, Fasshauer et al. 1998), which engage in SNARE domain interactions to form a highly stable tetra-helical super-coil (Kee et al. 1995, Sutton et al. 1998, Weninger et al. 2003).

In exocytosis, ternary SNARE complex formation involves two interacting partners at the target plasma membrane (t-SNAREs), a Qa-type syntaxin, a SNAP25 (synaptosomal-associated protein of 25kD)-like protein, which contains both a Qb- and Qc-type SNARE domain, and a R-type VAMP (vesicle associated protein), also called v-SNARE (vesicular SNARE, see Figure 1, Bonifacino and Glick 2004, Fukuda et al.

2000, Jahn and Grubmuller 2002, Jahn et al. 2003, Sollner et al. 1993a, Sollner et al. 1993b). In addition to their Qa SNARE domain, syntaxins are characterized by a regulatory helical bundle (H_{abc}) in their N-terminus (depicted in yellow color in Figure 1; Bracher et al. 2002). This N-terminal bundle is thought to exert an auto-inhibitory function by binding of the Qa-SNARE domain resulting in the so-called closed conformation (Dulubova et al. 1999, Hanson et al. 1995). In order to interact with cognate Qb-, Qc- and R-type SNARE domain containing proteins which is required for ternary complex formation, the syntaxin has to adapt an open and active conformation (see Figure 1; Bracher et al. 2002, Dulubova et al. 1999, Hanson et al. 1995). Conformational changes, assisted by accessory proteins (see below chapter I.2), allow for a dynamic equilibrium between the open and closed states and regulate syntaxin function (Figure 1 - 1; Gerst 2003, Jahn et al. 2003, Margittai et al. 2003).

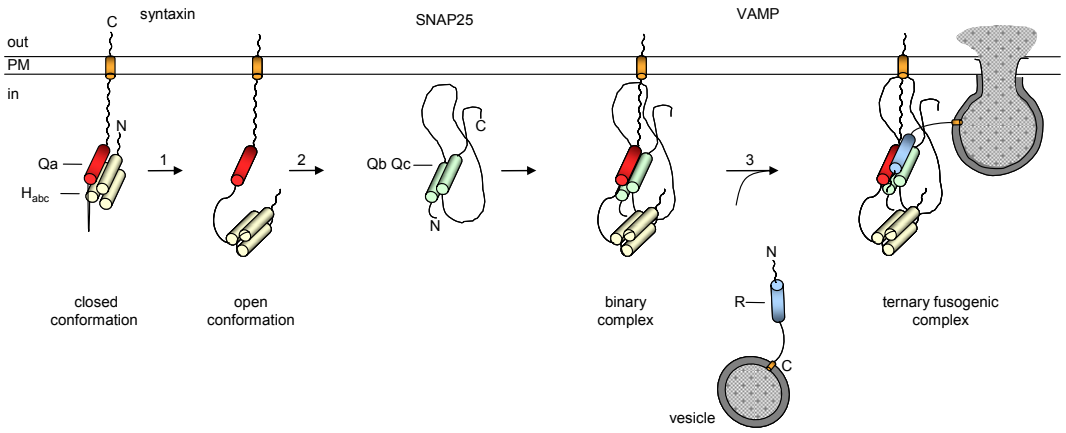


Figure 1. Proposed mechanism for SNARE protein mediated membrane fusion. Qa, syntaxin SNARE domain; Qb Qc, SNARE domains present in SNAP25; R-SNARE domain present in VAMP. (1) Dynamic equilibrium between open and closed conformation.(2) Binary complex formation of syntaxin and SNAP25. (3) Ternary complex formation upon binding a VAMP. For further details see text.

A current two-step model for SNARE-mediated vesicle fusion suggests that the open conformation of syntaxin and SNAP25 engage in a binary t-SNARE complex (shown in Figure 1, - 2). This serves as a docking station for the VAMP in the vesicle membrane, as depicted in Figure 1 - 3. Consequently a high affinity ternary SNARE complex is formed, tethering the vesicle to the target membrane (reviewed in Bonifacino and Glick 2004, Jahn and Grubmuller 2002, Jahn et al. 2003). SNARE complex formation has

been shown to follow a zippering mechanism, which starts from the N-terminus of the loosely paired SNARE domains, and is thought to account at least partially for the specificity of the fusion reaction (Fasshauer 2003, Pelham 2001, Pobbati et al. 2006, Sudhof 2004, Xue and Zhang 2002). Completion of the formation of the SNARE complex is thought to bring the vesicle in close proximity with the target membrane, and is considered to initiate fusion of the two opposing lipid bilayers, resulting in the secretion of the vesicle content (Jahn and Grubmuller 2002, Jahn et al. 2003, Jahn and Scheller 2006). The final fusion of the lipid bilayers and the release of the vesicle content may sometimes need additional (external) stimuli, e.g. Ca^{2+} influx (Jahn et al. 2003, Sudhof 2004, Südhof 1995).

Disbanding the ternary complex is energy consuming and requires the activity of NSF (*N*-ethylmaleimide sensitive factor) and α-SNAP (soluble NSF attachment protein), which allow for the recycling of the SNARE components after vesicle fusion (reviewed in Bonifacino and Glick 2004, Jahn et al. 2003).

I.2 SNARE protein function is tightly regulated

I.2.1 Regulation by accessory proteins

SNARE protein mediated vesicle fusion at target membranes is a highly coordinated process, requiring tight spatial-temporal regulation. A plethora of accessory and regulatory proteins have been shown to interact with all components of the SNARE vesicle fusion machinery in animals and yeast systems thereby regulating their function (Gerst 2003, Hong 2005, Jahn 2000). GTPases, ATPases and intracellular Calcium levels seem to be major players in controlling vesicle traffic and membrane fusion (Gerst 2003, Martin 2001, Nuoffer and Balch 1994, Spang 2002, Turner et al. 1999).

In addition, to the above components, conformational regulation of syntaxins is conserved in animals an yeast and is assisted by a distinct group of SNARE regulators, so-called SM (Sec1/Munc18) chaperone-like proteins, which are present in all eukaryotic genomes including plants (Gerst 2003, Li and Chin 2003, Toonen and Verhage 2003). In animals, SM proteins act in concert with Munc13 scaffold proteins and are supposed to modulate syntaxin function by direct protein-protein interactions (Li and Chin 2003, Toonen and Verhage 2003). Munc18-like proteins have been implicated in stabilizing the inactive closed conformation of syntaxins, while Munc13 is

thought to reverse Munc18-action and prime syntaxin for ternary complex formation (Gerst 2003, Toonen and Verhage 2003).

Loss-of-function mutants in these genes are often associated with severe phenotypes and several human immune disorders are associated with mutations in SNARE regulatory proteins (Stow et al. 2006, Toonen and Verhage 2003). A genetic defect in *Munc13-4* is the cause of a subtype of familial haemophagoytic lymphocytosis, a human immune deficiency characterized by severe hyperinflammatory syndrome with activated macrophages and T-lymphocytes (Stow et al. 2006). Mutations in the *Munc13* homolog of *C. elegans*, UNC13, cause strong pre-synaptic defects in neurotransmission resulting in a paralyzed organism (Richmond et al. 1999). Deletion of *Munc18-1* in mouse leads to a complete loss of neurotransmitter secretion from synaptic vesicles and results in early embryonic lethality (Verhage et al. 2000).

Interestingly, also *Arabidopsis* mutants lacking a functional copy of the SM gene *KEULE*, die early in development (Assaad et al. 2001, Waizenegger et al. 2000). Like animal SM proteins, KEULE was detected in soluble and in membrane-associated cellular fractions (Waizenegger et al. 2000). The *Arabidopsis* genome harbors six genes encoding SM-like proteins: *Sec1a, Sec1b, Keule, Vps33, Vps45, Sly1*. A similar number is present in the human genome (Hong 2005). It is not known whether SM proteins in plants function by regulating syntaxin conformation similar to animal SM proteins. Since plants appear to have an increased number of SNARE proteins compared to animals of similar genomic complexity, it seems possible that plants have evolved additional ways of regulating their surplus of vesicle trafficking components.

I.2.2 Regulation of syntaxin function by phosphorylation

Amongst the regulators of vesicle fusion, protein kinases and phosphatases have been identified to act on both SNARE proteins and their accessory proteins (Gerst 2003, Snyder et al. 2006). Syntaxin phosphorylation has been addressed in several studies in yeast and mammalian systems, as described in more detail below (Gerst 2003, Marash and Gerst 2003, Snyder et al. 2006, Turner et al. 1999). While studies from *Saccharomyces cerevisiae* report inhibition of SNARE complex formation by syntaxin phosphorylation at N-terminal residues (Gurunathan et al. 2002, Marash and Gerst 2003, Marash and Gerst 2001), phosphorylation of *Rattus norvegicus* syntaxin1a (*Rn*Stx1a, see alignment in Figure 7) at serine 14 or serine 188 did not counteract

fusogenic SNARE complex formation (Foletti et al. 2000, Risinger and Bennett 1999, Tian et al. 2003). It has been proposed that phosphorylation at N-terminal residues primes Stx1a for ternary complex formation (Snyder et al. 2006).

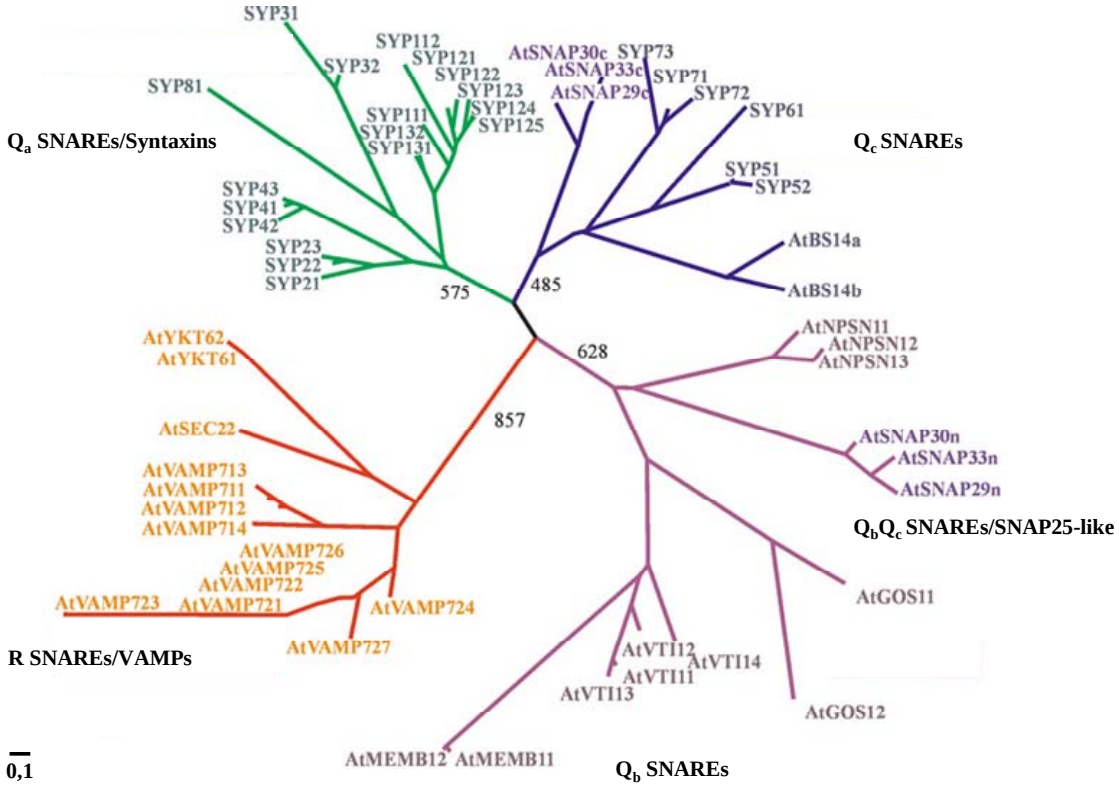
In plants, syntaxin phosphorylation has been reported to occur upon pathogen perception in basal defenses (Benschop et al. 2007, Nuhse et al. 2003) and in race-specific immunity (Heese et al. 2005). In *Arabidopsis* cultured cells, phosphorylation of the two closely related syntaxins SYP121 (also designated PEN1) and SYP122 at N-terminal serine residues, is induced by the bacterial elicitor peptide flg22, a potent inducer of basal defenses in plants (Benschop et al. 2007, Felix et al. 1999, Gomez-Gomez et al. 1999, Nuhse et al. 2003). Recently, a related syntaxin of tobacco, *Nt*SYP121, the proposed ortholog of PEN1, has been demonstrated to be phosphorylated in response to race specific signaling of the tomato *Cf9* gene in a heterologous test system (Heese et al. 2005). Interestingly, these syntaxins share potential N-terminal phospho-serine residues. However, the physiological role of these phosphorylation events remains elusive.

I.3 SNARE proteins are abundant in plant genomes and may exert specialized plant-specific functions

Genome wide analyses of SNARE genes in vertebrates, plants and other higher eukaryotes, has revealed an increase in the diversity of SNAREs compared to unicellular organisms and may reflect their complex multicellular lifestyle (Bock et al. 2001, Dacks and Doolittle 2002, Sanderfoot 2007, Sanderfoot et al. 2000).

This increase in vesicle trafficking components appears to be even more pronounced in plants than in other higher eukaryotes. The *Arabidopsis* genome contains 64 SNARE protein family members (Figure 2), in poplar there are 74 and in rice 60, while the human genome harbours 39 genes encoding SNARE trafficking components (Bock et al. 2001, Sanderfoot 2007, Sanderfoot et al. 2000). This is mostly due to an extension in the number of presumably secretory SNAREs of the syntaxin and VAMP type (Sanderfoot 2007). The SYP1 (syntaxin of plants 1) group of plasma-membrane resident syntaxins and R-SNAREs of the VAMP7-clade in green plants show most evolutionary diversification, as can be seen from the phylogenic tree of *Arabidopsis* SNAREs depicted in Figure 2. Members of the VAMP72 sub-clade appear to be

specific to land plants and have been speculated to be involved in specialized secretory functions (Sanderfoot 2007, Uemura et al. 2004).



Graphic modified from Uemura et al 2004

Figure 2. Unrooted phylogenic tree of *Arabidopdsis* SNARE proteins (modified from Uemura et al. 2004). The construction of the tree is based on amino acid residues in the SNARE domain. Numbers at three branches represent bootstrap values with 1000 replications. The scale bar indicates the Dayhoff distance among the SNARE molecules. Note the apparent diversification among members of the SYP1 and the VAMP72 group. Green, Qa-SNARE/Syntaxin; purple, Qb-SNARE; blue, Qc-SNARE; red, R-SNARE.

In contrast, *SNAP25*-like SNARE homologues are present in similar numbers in higher plants and animals. Four genes encoding *SNAP25*-like SNAREs have been annotated in the human genome. The same number of genes is present in poplar and three, *SNAP29, SNAP30,* and *SNAP33*, are found in the *Arabidopsis* genome (see Figure 2; Sanderfoot 2007, Sanderfoot et al. 2000, see Figure 2; Uemura et al. 2004). Gene expression studies indicate that *SNAP33* is the only *SNAP25*-like gene of *Arabidopsis* expressed in

significant amounts in vegetative tissue (Wick et al. 2003; C. Neu, unpublished). It is therefore considered to be involved in the majority of intracellular vesicle fusion events.

In parallel to an increase in SNARE numbers and SNARE protein diversity, some subunits of the exocyst complex, which has been proposed to act as initial tethering complex to specify the site of vesicle docking before the action of the SNARE protein machinery (Hsu et al. 1999), seem to be highly diversified in plants (Elias et al. 2003). Twenty-three *Arabidopsis* genes encode for a potential EXO70 subunit although only one *EXO70* gene is present in worms, flies and the humans (Elias et al. 2003). This raises the possibility that the abundance of genes related to exocytosis and secretion in plants might reflect a greater level of functional diversification and/or the engagement of vesicle trafficking in plant-specific processes (Sanderfoot 2007, Sanderfoot et al. 2000).

Indeed, several plant SNAREs, including members of the SYP1 syntaxin group, have been assigned to plant-specific processes. The tobacco syntaxin *Nt*SYP121 functions in hormone-dependent ion channel regulation (Leyman et al. 1999) and in *Arabidopsis* KNOLLE syntaxin (SYP111) and its accessory protein KEULE play a role in cytokinesis (Lauber et al. 1997, Waizenegger et al. 2000). The SNARE proteins *At*VAM3 and *At*VTI11 are important for gravity-sensing (Yano et al. 2003), and the syntaxin *At*SYP61 is involved in salt tolerance (Zhu et al. 2002).
Furthermore, several syntaxins have been implicated in plant-microbe interactions. *Nicotiana benthamiana Nb*SYP132 as well as *Arabidopsis* SYP121 (PEN1) and barley *Hv*Ror2 (Assaad et al. 2004, Bhat et al. 2005, Collins et al. 2003, Lipka and Panstruga 2005), have been demonstrated to play a role in plant defence responses. Very recently, the first evidence for a role of SNARE proteins in symbiosis has also been presented, in a study on the subcellular localization of the *Medicago truncatula* syntaxin *Mt*SYP132 (Catalano et al. 2007).

I.4 Vesicle trafficking events contribute to a first line of plant defenses

Plants are able to sense pathogens either through pattern recognition immune receptors (also designated PAMP-, pathogen associated molecular pattern, receptors), which are similar to Toll-like receptors in animal immunity (Gomez-Gomez and Boller 2002, Hammond-Kosack and Parker 2003, Robatzek 2007, Zipfel et al. 2006, Zipfel et al. 2004) or via mostly intracellular resistance (*R*)-gene products, which mediate race-specific recognition of pathogen encoded *Avr* (avirulence) gene products and resemble CATERPILLAR/Nod proteins of the animal innate immune system (Dangl and Jones 2001, Fritz et al. 2006, Holt et al. 2003, Mariathasan and Monack 2007, Nimchuk et al. 2003). Several genes encoding *Arabidopsis* PAMP receptors, i.e. the *FLS2* kinase which recognizes the flg22 peptide derived from bacterial flagellin (Felix et al. 1999, Gomez-Gomez and Boller 2002, Gomez-Gomez et al. 1999) and *EFR*, a PAMP receptor sensing the bacterial elongation factor EF-Tu (Zipfel et al. 2006), and a gene encoding the receptor for the well-characterized fungal elicitor xylanase from *Trichoderma viride* (Bailey et al. 1992, Fuchs et al. 1989), *LeEix2,* has been molecularly isolated and characterized from tomato (*Lycopersicon esculentum*; Ron and Avni 2004). Downstream signaling events following PAMP receptor or *R*-gene activation are thought to initiate cellular reprogramming to either stop the pathogen before successful invasion, or kill the invaded cell (and the pathogenic invader) by a localized hypersensitive (HR) cell death response (Glazebrook 2001, Heath 2000). Systemic signaling through salicylic acid (SA), as well as jasmonic acid (JA) /ethylene (ET) based signals is initiated and is thought to prevent secondary infections in distant tissues (Beckers and Spoel 2006, Durrant and Dong 2004, Glazebrook 2005, Grant and Lamb 2006). While systemic acquired resistance is thought to be effective against biotrophic parasites and was shown to act antagonistically to JA/ET based signals, the latter are thought to restrict necrotrophic pathogens and herbivores (Beckers and Spoel 2006, Glazebrook 2001, Grant and Lamb 2006).

To block microbial infection at the cell periphery, secretory vesicles are expected to deliver cell wall material, cytotoxic compounds and pathogenesis related (PR) proteins with antimicrobial activity to the site of attack to prevent invasion (Farmer et al. 2003, Robatzek 2007, Schulze-Lefert 2004, Van Loon and Van Strien 1999). Callose-

containing cell wall appositions (papillae) are thought to constitute a physical barrier against invading pathogens and reinforce the cell wall at sites of wounding (Bushnell and Bergquist 1974, Jacobs et al. 2003, Nishimura et al. 2003). Indeed, vesicle trafficking events towards the site of pathogenic attack can be observed microscopically and have been described for many interactions between plants and non-beneficial microbes (Huckelhoven 2007, Schmelzer 2002). Upon pathogen attack, plant cells display numerous structural re-arrangements focused towards the attempted fungal entry site involving the cytoskeleton and the cytoplasm as well as organelles (Kobayashi and Hakuno 2003, Koh et al. 2005, Lipka and Panstruga 2005, Schmelzer 2002, Takemoto et al. 2003, Takemoto et al. 2006). The focal delivery of vesicles containing cell wall material and cytotoxic defense compounds is believed to be part of a first line of inducible plant defenses upon pathogen encounter (Lipka and Panstruga 2005, Robatzek 2007).

I.4.1 PEN1 mediates pre-invasion resistance at the cell periphery

In a genetic screen for *Arabidopsis* mutants displaying enhanced invasion or "penetration" of the non-adapted barley powdery mildew *Blumeria graminis* f.sp. *hordei* (designated *B. g. hordei* further on; Collins et al. 2003) four independent alleles of *pen1(penetration 1)* have been isolated from an ethyl methane sulfonate (EMS) mutagenized population each supporting enhanced fungal entry of *B. g. hordei* conidiospores. This provided for the first time genetic evidence for the possible existence of a vesicle-based and secretory disease resistance mechanism at the cell periphery, mediated by a single syntaxin family member. Resistance to non-adapted parasites, so-called host resistance, is the most common and durable form of plant defense responses (Nurnberger and Lipka 2005, Thordal-Christensen 2003). It is defined as resistance of an entire plant species to all isolates of a microbial species and consequently restricts the host range of a potential plant pathogen to a narrow set of plants species (Ellis 2006, Holub and Cooper 2004, Nurnberger and Lipka 2005).

On *Arabidopsis* (and other non-host plants) the barley powdery mildew fungus fails to complete its life cycle. Usually, the infection is stopped at the pre-invasion stage, coincident with the formation of cell wall appositions, so-called papillae and the majority of attacked cells survive (Collins et al. 2003). Papilla form underneath both,

the primary germ tube of the fungus and the appressorial germ tube (or appressorium), which potentially develops an invasive feeding structure, the haustorium, within 10-12 hours post inoculation (hpi; Thordal-Christensen et al. 2000). The formation of callose-containing cell wall appositions underneath fungal appressoria has been suggested to constitute a physical barrier against fungal invasion (Bushnell and Bergquist 1974, Jacobs et al. 2003, Nishimura et al. 2003). Rarely, in less than 20% of the interaction sites on *Arabidopsis*, fungal growth is terminated post-invasively, after a haustorial initial was accommodated, thus pre-invasion defenses are fast and function effectively within the first 12 hours of pathogenesis (Collins et al. 2003, Lipka et al. 2005).

In *Arabidopsis* plants lacking a functional copy of *PEN1* pre-invasive non-host resistance responses are severely impaired. *pen1*-1 null mutant plants display fungal entry rates of more than 60% while wild-type plants support less then 20% of *B. g. hordei* ingress. It has been demonstrated that the formation of callose-containing cell wall appositions (papillae) is significantly delayed in *pen1*-1 compared to wild-type plants, suggesting that PEN1 may be required for the rapid delivery of cell wall material to the periphery (Assaad et al. 2004). Further molecular components of non-host resistance processes were identified from three other mutants with phenotypes similar to *pen1,* designated *pen2, pen3* and *pen4*. The affected genes were molecularly isolated and encode for a ß-glycosyl hydrolase, an ABC-type transporter and a γ-glutamylcysteine synthethase (Lipka et al. 2005, Stein et al. 2006, M. Lim and S. Somerville unpublished). Epistasis analysis suggested that *PEN1* acts in a vesicular defense pathway leading to papilla formation distinct from responses mediated by *PEN2* to *PEN4* (Ellis 2006, Lipka et al. 2005). How these pathways are connected and individual components are activated is the focus of ongoing research projects.

I.4.2 SNAP33 and VAMP722 are potential components of putative PEN1 containing ternary SNARE complexes

Similar to *pen* mutants in *Arabidopsis*, barley plants lacking a functional copy of the *HvRor2* syntaxin fail to mount efficient broad-spectrum disease resistance responses to *B. g. hordei* isolates (Freialdenhoven et al. 1996). *Arabidopsis PEN1* was demonstrated to be the functional homolog of barley *HvRor2* in a transient single cell expression system (Collins et al. 2003). Broad-spectrum disease resistance to *B. g. hordei* in barley,

which effectively prevents fungal entry into host epidermal cells, is mediated by recessively inherited *Hvmlo* alleles (for review see Panstruga 2005). The seven transmembrane plasma membrane-resident *Hv*MLO wild-type protein is thought to act as a negative regulator of disease resistance responses to powdery mildews (Panstruga 2005, Panstruga and Schulze-Lefert 2003).

Like barley *Hvmlo*-resistant mutants, recessive mutations in the recently identified *Arabidopsis* functional *HvMlo* homolog, *AtMLO2*, result in resistance to the adapted *Arabidopsis* powdery mildews *Golovinomyces orontii and Erysiphe cichoracearum* (Consonni et al. 2006). In both, barley and Arabidopsis *mlo* mutants, fungal pathogenesis is terminated at the plasma membrane prior to invasion of leaf epidermal cells, suggesting a conserved *mlo*-mediated resistance mechanism between monocotyledonous and dicotyledonous species. Consistent with the suppression of *Hvmlo*-based resistance by a mutation in the *HvRor2* syntaxin in barley (Freialdenhoven et al. 1996), mutations in *PEN1* suppress *mlo2*-mediated powdery mildew resistance in *Arabidopsis* (Consonni et al. 2006). However, not only loss of *PEN1* but also loss of *PEN2* or *PEN3,* which are thought to act in a pathway from distinct *PEN1*, suppresses *mlo2*-mediated pre-invasion resistance suggesting that *HvMlo/MLO2* might be a general regulator of pre-invasion resistance upstream of *PEN1, PEN2,* and *PEN3* (Consonni et al. 2006). For this reason, and because *mlo*-based resistance has been demonstrated to be durable and effective against all known powdery mildew isolates (Buschges et al. 1997, Jorgensen 1992) and is independent of SA-and JA/ET- signaling (Consonni et al. 2006), *mlo*-based resistance has been speculated to be mechanistically identical to non-host resistance (Humphry et al. 2006).

Arabidopsis PEN1, as well as barley *Hv*Ror2 and *Hv*Mlo proteins, have been shown to focally accumulate underneath the attempted fungal entry sites (Assaad et al. 2004, Bhat et al. 2005). Furthermore, using yeast two hybrid analysis and *in planta* FRET imaging of fluorophore-tagged protein versions, *Hv*Mlo has been shown to physically interact with *Hv*Ror2 in *B. g. hordei*-triggered micro-domains and as well as in the plasma membrane of healthy leaf epidermal cells (M. Kwaaitaal, R. Bhat and C. Consonni, unpublished). For this reason it has been postulated that *Hv*Mlo orthologs in barley and *Arabidopsis* might regulate *Hv*Ror2/PEN1 syntaxin activity (Panstruga 2005, Schulze-Lefert 2004).

In addition to the syntaxins PEN1 and *Hv*Ror2 and their potential regulator Mlo/*At*MLO2, a SNAP25 homologue, *Hv*Snap34, was shown to be required for full *mlo*-mediated resistance to *B. g. hordei* (Collins et al. 2003). *Hv*Snap34 was demonstrated to interact with *Hv*Ror2 syntaxin in yeast two hybrid experiments pointing to the existence of potential binary and ternary PEN1/*Hv*Ror2-containing SNARE complexes involved in disease resistance to powdery mildews (Collins et al. 2003). In *Arabidopsis* three genes encoding for SNAP25-like SNARE protein are present, *SNAP29*, *SNAP30* and *SNAP33* (see phylogenic tree Figure 2, chapter I.3). *SNAP29* is mainly expressed in pollen and *SNAP30* is expressed primarily in radicles and roots. *SNAP33* expression, however, is detected to a high amount in all tissues (Wick et al. 2003, C. Neu unpublished) rendering it the most likely candidate involved in a potential SNARE complex mediating resistance responses in *Arabidopsis*. Indeed, a putative pathogen induced ternary complex containing SNAP33 and PEN1 could be detected in total protein extract from *B. g. hordei* challenged *Arabidopsis* leaf material (Kwon et al. in preparation).

To date, no genetic evidence has been published proving the engagement of a R-type SNARE or VAMP in PEN1/Ror2-dependent defence responses. Confocal imaging using various fluorochrome-tagged variants of plant plasma-membrane resident VAMPs has revealed that barley *Hv*Vamp722 and its homologue VAMP722 and the highly sequence-related VAMP721 of *Arabidopsis* focally accumulate at fungal entry sites (S. Bau, C. Neu, N. Clemens unpublished). Therefore, VAMP721/VAMP722 SNAREs represent candidate partners of a putative resistance-mediating ternary SNARE complex, containing VAMP721/VAMP722 PEN1/Ror2, Snap34/SNAP33.
In vitro, PEN1, SNAP33 and members of the VAMP72 group engage in ternary SNARE complexes (Kwon et al. in preparation). Interestingly, *pen1*-3, a partial defect allele of PEN1 initially observed by Collins et al., which carries a glycine to aspartate exchange in the conserved SNARE domain of the protein (see alignment in Figure 7, chapter III), displayed a selective defect in ternary complex formation with VAMP722 but not with other members of the VAMP72 group tested. Furthermore, simultaneous transcript-based depletion of both, *VAMP721* and *VAMP722* in transgenic *Arabidopsis* plants revealed enhanced *B. g. hordei* entry in leaf epidermal cells similar to the *pen1*-1 phenotype (Kwon et al. in preparation). This was not seen in plants lacking either *VAMP721* or *VAMP722* which indicates that these genes have overlapping and

redundant functions. It is conceivable that *in planta* VAMP721 and VAMP722 might both engage in PEN1-containing SNARE complexes (Kwon et al. in preparation).

I.4.3 PEN1 and SYP122: Functional diversity or genetic redundancy?

Based on sequence identity *SYP122* is the closest relative of *PEN1* among the 18 syntaxin encoding genes in the *Arabidopsis* genome. At the protein level these two share 63% identical residues (see phylogenic tree in Figure 2 and alignment in 0, chapter III). Despite this high sequence identity, SYP122 and PEN1 have diversified functions in plant immunity (Assaad et al. 2004). Although SYP122 accumulates underneath fungal appressoria at attempted penetration sites and *SYP122* transcript levels are highly pathogen responsive, *syp122* mutant plants retain wild-type-like pre-invasion resistance to the non-adapted powdery mildew fungus *B. g. hordei* and papilla formation appears to be normal (Assaad et al. 2004). Interestingly, in cultured *Arabidopsis* cells both proteins appear to be phosphorylated at N-terminal serine residues in response to bacterial elicitation, pointing to a potential pathogen-induced co-regulation at the post-translational level (Benschop et al. 2007 and see chapter I.2.2. for details). It remains to be clarified whether differences in the gene expression of *PEN1* and *SYP122,* e.g. steady state or inducible protein abundance, or diversified regions in the amino acid sequence of both proteins account for their differential requirement in disease resistance.

Interestingly, *pen1/syp122* double mutant lines are severely dwarfed and necrotic, suggesting that SYP122 and PEN1 may exert additional partially overlapping functions (Assaad et al. 2004). In a recent publication the *pen1/syp122* double mutant was shown to exhibit multicellular death responses upon challenge with both, the non-adapted powdery mildew *B. g. hordei* and the adapted *E. cichoracearum* reminiscent of hypersensitive like cell death responses known to be elicited by race-specific resistance (*R)* gene signaling (Glazebrook 2001, Pontier et al. 1998, Zhang et al. 2007). Furthermore *pen1* and *syp122* single mutants showed wild-type-like responses to virulent *Pseudomonas syringae* pv *tomato* indicating that the multicellular death response observed with powdery mildew pathogens was not generally active in the *pen1*-1 mutant in response to other plant pathogen, i.e. the tested *Pseudomonas* strain (Zhang et al. 2007). Additionally, Zhang et al reported elevated levels of the defense signaling molecule SA in the *pen1*-1 single mutant and massive SA accumulation in the

pen1/syp122 double mutant in unchallenged tissue and increased transcription of the defense-response gene *PDF1.2,* which is indicative of an activation of ET and JA pathway (Manners et al. 1998, Penninckx et al. 1998), was detected (Zhang et al. 2007). These findings lead to the conclusion that both syntaxin might have regulatory functions in both SA and its antagonistic ET/JA pathway (Spoel et al. 2003) in response to powdery mildews.

A recent genome-wide analysis of plant SNAREs has identified Qa-SNARE members highly sequence related to PEN1 and Ror2 but not to SYP122 in the genomes of rice and poplar (Sanderfoot 2007). The deduced amino acid sequence of rice *Os*Ror2 (Os03g57310) is 83% identical to the barley Ror2 protein. In poplar, two genes, designated *PtPen1a* (Poptr1_1:256235) and *PtPen1b* (Poptr1_1:802113) are highly sequence related to *PEN1* (Sanderfoot 2007). The presence of two potential homologues of *PEN1* in poplar might reflect a recent gene duplication event. Since *SYP122* is absent from the poplar and rice genomes, one may speculate that during evolution gene duplication and diversification of *PEN1* has given rise to *SYP122* in *Arabidopsis*. Such a scenario could explain partial redundancy paralleling functional diversity in both genes.

I.5 Aim of the project

The PEN1 syntaxin has been shown to contribute to plant immune responses to adapted and non-adapted ("non-host") powdery mildew fungi (Collins et al. 2003, Consonni et al. 2006). Focal transport of vesicles to the site of fungal attack (Schmelzer 2002) coincides with the accumulation of PEN1 underneath fungal infection structures (Assaad et al. 2004, Bhat et al. 2005, Schmelzer 2002). Therefore it is likely that PEN1 is part of a vesicle associated resistance mechanisms at the cell periphery; presumably by guiding toxic defense vesicles to the site of fungal attack (Kwon et al. in preparation, Panstruga 2005, Schulze-Lefert 2004). The identification of PEN1 SNARE partner proteins involved in these processes is currently in process (Kwon et al. in preparation). This project was designed to assess structural and functional conservation of the PEN1 protein involved in vesicle associated immune responses.

Three different experimental strategies were followed in parallel. One strategy aimed to provide insight in the structure-function relationship of PEN1 by targeting distinct conserved amino acids (see chapter III.1. and III.2.). In a second set of experiments, I addressed the question how functional differences between the two highly sequence-related syntaxin proteins PEN1 and SYP122 are established (see chapter III.3.). To this end, I exchanged the entire N-terminal domain of PEN1 with the corresponding domain of its closest relative SYP122 to generate a chimeric syntaxin. In addition, differences in protein expression between PEN1 and SYP122 were analyzed. The third approach aimed to analyze the localization of presumed SNARE partner proteins of PEN1 during pathogenesis and in unchallenged tissue (chapter III.4).

II Material and Methods

II.1 Materials

II.1.1 Plant material

The mutants and transgenic plants observed and generated in this study were in the genetic background of the *A.thaliana* accession Columbia-0 (Col-0), as it is listed in Table 1.

Table 1. *Arabidopsis thaliana* mutants and transgenic *Arabidopsis thaliana* lines

Ecotype	Mutation	Transgene	Reference
Col-0	-	-	Nottingham Arabidopsis Stock Center (NASC)
Col-0	*pen1*-1	-	Collins et al 2003
Col-5 (Col-0 *gl1*)	*pen1*-3	-	Collins et al 2003
Col-0	*pen1*-1	p35S::GFP-PEN1	Collins et al 2003
Col-0	*syp122*-1	-	SALK_008617, Assaad et al 2003
Col-0	*snp33-1*	-	SALK_075519
Col-0	*vmp722*-1	p35S::mYFP-VAMP722	C.Neu, personal communication
Col-0	*mlo2*-11	-	Consonni et al 2006
Col-0	*mlo2*-11/*pen1*-1	-	Consonni et al 2006

II.1.2 Plant pathogens

The barley powdery mildew, *Blumeria graminis* forma specialis. *hordei* isolate K1 (Shen 2004), designated *B. g. hordei* further on, was used for spore inoculation experiments (see section II.2.1.3).

The *Arabidopsis* powdery mildew *Erysiphe cichoracearum* strain UCSC1 (Koh et al. 2005), was used for pilot experiment by our collaborator M.Lim at the Standford laboratory.

II.1.3 Bacterial and yeast strains

Escherichia coli strains were obtained from Invitrogen (Invitrogen, Karlsruhe, Germany). For plasmid amplification DH5α was used. Plasmid DNA containing the full length cddB gene containing Gateway cassette was amplified in Db3.1 cells, resistant to the cddB gene product (Invitrogen, Karlsruhe, Germany). The *Agrobacterium tumefaciens* strain GV3101 was used for stable *Arabidopsis thaliana* transformation (Koncz and Schell 1986).
Saccharomyces cerevisiae strain JD53 (Dohmen et al. 1995) was used for the Split Ubiquitin two-hybrid assay, see below section II.2.3).

Table 2. Bacterial and yeast strains

Bacterium	strain	genotype	Reference/source
E.coli	DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Invitrogen, Karlsruhe, Germany
E.coli	Db3.1	F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1	Invitrogen, Karlsruhe, Germany
A.tumefaciens	GV3101	pMP90RK (Gm^R, Km^R, Rif^R)	Koncz and Schell, 1986
S.cerevisiae	JD53	MATα his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ63 ura3-52	Dohmen et al. 1995

II.1.4 Enzymes

II.1.4.1 Restriction enzymes

Restriction enzymes were bought from New England Biolabs (Frankfurt, Germany) or Roche (Mannheim, Germany) and were used according to the manufacturer's reaction conditions in the provided reaction buffers.

II.1.4.2 Other enzymes

Commercially available enzymes were used with the provided buffers under reactions conditions specified in the manufacturer's manual. Home-made *Taq* polymerase was used in the buffer supplied with the Roche enzyme.

Table 3. Other enzymes

Name	Source
Lysozyme	Roth, Karlsruhe, Germany
Mung Bean Nuclease	New England Biolabs, Frankkfurt, Germany
RNAse	Sigma, Steinheim, Germany
Pfu polymerase	Stratagene, Heidelberg, Germany
Taq polymerase	Roche, Mannheim, Germany
Taq polymerase	home-made
T4 DNA Ligase	Roche, Mannheim, Germany
Shrimp Alkaline Phosphatase	New England Biolabs, Frankfurt, Germany

II.1.5 Antibodies

H. Thordal-Christensen provided the PEN1-antiserum and T. Nühse provided the anti-serum against SYP122, other antibodies were commercially available and purchased from the in Table 4 indicated manufacturer.

Table 4. Primary and secondary antibodies

Name	Source	conjugate	Dilution*	Reference
PEN1-antiserum	rabbit, polyclonal	-	1:1000	H.T-Christensen, KVL, DK
SYP122-antiserum	rabbit, polyclonal	-	1:5000	Nühse et al. 2003
α-HA	rat, monoclonal	-	1:5000	Roche, Mannheim, Germany
α-rat	rabbit	HRP	1:10 000	Sigma, Steinheim, Germany
α-rabbit	goat	HRP	1:10 000	Santa Cruz, Santa Cruz, CA,USA

* in 1x TBS-T

II.1.6 Antibiotics

Stock solutions of the antibiotics were prepared at the given concentrations and stored at -20°C (see Table 5). The antibiotics were purchased from various companies as listed in Table 7.

Table 5. Antibiotics

Name	Concentration *	Source
Ampicillin	100 mg/ml in dH_2O	Roth, Karlsruhe, Germany
Carbenicillin	50 mg/ml in dH_2O	Sigma, Steinheim, Germany
Kanamycin	50 mg/ml in dH_2O	Serva, Heidelberg, Germany
Rifampicin	50 mg/ml in DMSO	Fluka, Buchs, Switzerland

*1000 x stock in in dH_2O

II.1.7 Oligonucleotides

Oligonucleotides used in this study are listed in Table 6.

Table 6. Oligonucleotides

Name	Oligonucleotide sequence	Used for	Orientation
SeqL-A	TCGCGTTAACGCTAGCATGGATCTC	Sequence pDONR201 (Invitrogen)	forward
SeqL-B	GTAACATCAGAGATTTTGAGACA	Sequence pDONR201 (Invitrogen)	reverse
Spa019	GAACGATTTGTTTGCGGCGGCGTTCTCTCGC TTCC	$PEN1^{S6A\ S7A\ S8A}$	forward
Spa020	GGAAGCGAGAGAACGCCGCCGCAAACAAAT CGTTC	$PEN1^{S6A\ S7A\ S8A}$	reverse
Spa021	GAACGATTTGTTTGCGAGCTCATTCTCTCG	$PEN1^{S6A}$	forward
Spa022	CGAGAGAATGAGCTCGCAAACAAATCGTTC	$PEN1^{S6A}$	reverse
Spa023	CGATTTGTTTTCCGCGTCATTCTCTCGC	$PEN1^{S7A}$	forward
Spa024	GCGAGAGAATGACGCGGAAAACAAATCG	$PEN1^{S7A}$	reverse
Spa025	GATTTGTTTTCCAGCGCGTTCTCTCGCTTC	$PEN1^{S8A}$	forward
Spa026	GAAGCGAGAGAACGCGCTGGAAAACAAATC	$PEN1^{S8A}$	reverse
Spa027	GAACGATTTGTTTGACGACGACTTCTCTCGC TTCC	$PEN1^{S6D,\ S7D,\ S8D}$	forward

Table 6 continued

Name	Oligonucleotide sequence	Used for	Orientation
Spa028	GGAAGCGAGAGAAGTCGTCGTCAAACAAATCGTTC	PEN1$^{S6D, S7D, S8D}$	reverse
Spa029	GAACGATTTGTTTGACAGCTCATTCTCTCG	PEN1^{S6D}	forward
Spa030	CGAGAGAATGAGCTGTCAAACAAATCGTTC	PEN1^{S6D}	reverse
Spa031	CGATTTGTTTTCCGACTCATTCTCTCGC	PEN1^{S7D}	forward
Spa032	GCGAGAGAATGAGTCGGAAAACAAATCG	PEN1^{S7D}	reverse
Spa033	GATTTGTTTTCCAGCGACTTCTCTCGCTTC	PEN1^{S8D}	forward
Spa034	GAAGCGAGAGAAGTCGCTGGAAAACAAATC	PEN1^{S8D}	reverse
Spa051	CCCGGAATTCATGGTGAGCAAGGGCGAGG	Add EcoRI to 5'mYFP	forward
Spa052	CAAACTGCAGCTTGTACAGCTCGTCCATGCCG	Add PstI to 3'mYFP	reverse
Spa053	GGACGCGTCGACATGGTGAGCAAGGGCGAGG	Add SalI to5'mYFP	forward
Spa054	GTTACGCGTCGACCTTGTACAGCTCGTCCATGC	Add SalI to 3'mYFP	reverse
Spa068	CGGATGAACGAACCGCGGCGCGACTGATTTCCAC	PEN1$^{L185A, D186A}$	forward
Spa069	GTGGAAATCAGTCGCGCCGCGGTTCGTTCATCCG	PEN1$^{L185A, D186A}$	reverse
Spa070	CTCAGCTTGATGACGCGGAGAGTCATGTGG	PEN1^{I255A}	forward
Spa071	CCACATGACTCTCCGCGTCATCAAGCTGAG	PEN1^{I255A}	reverse
Spa074	GGGGCGCGCCCGACCACACGCATTGTTAAC	Add 5'AscI to promoter PEN1	forward
Spa075	CCGAATTCCGAAAAATAGAGAAATC	Add 3'EcoRI to promoter PEN1	reverse
Spa076	CCAAGCTTCGAAAAATAGAGAAATC	Add 3'HindIII to promoter PEN1	reverse
Spa079	GTCATGTGGGTCGAGTTAGCTCCTTTGCGAGAGGCGGAAC	PEN1$^{A262V, I266A}$	forward
Spa080	GTCATGTGGGTCGACGCAAAGGAGCTAACTCGACCCACATGAC	PEN1$^{A262V, I266A}$	reverse
Spa081	CATAGCCCAGTCTTTGAGCACC	Sequence PEN1 promoter in pGUS rev	reverse
Spa082	CTGGAGAGAGTGAGAGATTC	Sequence PEN11 3'cds	forward
Spa088	CCGCTCGAGGACGTCGCCACCATGGTGAGCAAGGGCGAGGAG	Add XhoI AatII cosacATG-to 5'mYFP	forward
Sbi048	AGCACAAGTTTTATCCGGCC	In Cmr gene, for sequencing of Gateway cassettes	forward
Spa092	CAAAACTGGTGAAACTCAC	On CmR in GW cassette	reverse
Spa095	CATGATGCGGTTAAAGACGCGGCGAAGAATCTCAGGGAGC	PEN1$^{I227A, E228A}$	forward
Spa096	GCTCCCTGAGATTCTTCGCCGCGTCTTTAACCGCATCATG	PEN1$^{I227A, E228A}$	reverse

Table 6 continued

Name	Oligonucleotide sequence	Used for	Orientation
Spa097	CCGCTCGAGGACGTCCGAAAAATAGAGAAA TC	Add 3'AatII XhoI to pPEN1	reverse
Spa098	ACGCGTCGACGTGAGCAAGGGCGAGGAGC	Add 5'SalI and cut ATG from MYFP	forward
Spa100	GAGAGTGTTTCGTTGAGC	Sequence PEN1 5'	reverse
Spa101	GTTACGCGTCGACCCCTTGTACAGCTCGTCC ATGC	Add SalI 2xC to 3'mYFP	reverse
Spa102	GGACGAGCTGTACAAGGGGTCGAGAAAGAG GATCC	Add GG to pAM PAT mYFPs	forward
Spa103	GGATCCTCTTTCTCGACCCCTTGTACAGCTC GTCC	Add GG to pAM PAT mYFPs	reverse
P35S	ACAATCCCACTATCCTTC	on 35S promoter	forward
Spa109	GGACACGCTGAACTTGTGG	Sequence promoter in pAM PAT	reverse
Bü979	CTGTCAGTTCCAAACGTAAAACG	On NosP, for sequencing gene promoter in pAM PAT	forward
Spa113	CATAACCTTCAAGCTCCTG	Sequence snap33 5'cds	reverse
Spa115	GGATGATAAACTTAAAGTATC	On pPEN1	forward
Spa116	CGATTTGTTTTCCGGCTCATTCTCTCGC	$PEN1^{S7G}$	forward
Spa117	GCGAGAGAATGAGCCGGAAAACAAATCG	$PEN1^{S7G}$	reverse
Spa122	GCGCCTCGCCGTCTTAAGCGC	Sequence 5' of SYP122	reverse
Cn223	GCGTGGACCGCTTGCTGCAACT	LBb1 SALK left border T-DNA primer	n.a.
Cn224	TGGTTCACGTAGTGGGCCATCG	LBa1 SALK left border T-DNA primer	n.a.
Sbk114	AAGGTTTATTAGTAGTAACCAATCC	Snap33	forward
Sbk115	CTCTGATAAGCATCAGCTGATTCGG	Snap33	reverse
Spa129	GTATTCATGTCCCCTATACTAGG	On 5' GST	forward
Spa130	CTTAGCAAGCTACCTGAAATGC	On GST	forward
Spa131	GCATTTCAGGTAGCTTGCTAAG	On GST	reverse
Spa134	CCGCCGCCGGGATCACTC	On 3' mYFP for sequencing	forward
Sbk121	TCAACGCAATAGACGCCTTGCCTGAGG	On 5' PEN1 for sequencing	reverse
Spa135	GGGACGTCGGCTTAAGAGATCTTCGATTTTG	Add AatII to pSYP122	forward
Spa136	TTGGCGCGCCAAAAAGTAGAATCCGTAAAT	Add AscI to pSYP122	reverse
Spa145	GGAAGAAGCTGAAGGATGAGATGG	Sequence central SYP122	forward
Spa146	GGGGACCACTTTGTACAAGAAAGCTGGGT CCTTTCCAAGCAAACGGCGACCACG	3'SNAP33 without STOP, GW-extension	reverse
Sbk30	GGCTCAATGTTTGGTTTAAGGAAATCACCGG	Snap33 5'GW extension	forward
Spa151	CGCCTACTTGAATCTGTACG	On PEN1 5'UTR	forward

Table 6 continued

Name	Oligonucleotide sequence	Used for	Orientation
Spa154	CAACGCAATAGACGCCTTGCC	On 3'PEN1	reverse
Dom019	CAACGAAACACTCTCTTCATGTCACGC	CAPS *pen1*-1	forward
Spa156	CCTTGTCTTGTATCAATTGC	Upstream of NUI	forward
CAPS R	CATCAATTTCTTCCTGAGAC	CAPS *pen1*-1	reverse

n.a. not applicable

The oligonucleotides were purchased from metabion (Martinsried, Germany), operon biotechnologies (Cologne, Germany) and Sigma Genosys (Steinheim, Germany).

II.1.8 Chemicals and reagents

Laboratory grade chemicals and reagents used are listed in Table 7.

Table 7. Laboratory grade chemicals and reagents

Name	specification	Source
Acrylamid	Rotiphorese ® Gel 30	Roth, Karlsruhe, Germany
Aniline Blue	-	Sigma, Sterinheim, Germany
Bactoagar	BactoTM Agar	Becton, Dickinson and Company, Sparks, USA
BASTA	Basta	Bayer Crop Science, Monheim, Germany
Bradford reagent	-	Bio-Rad, Hercules, CA, USA
Carrier DNA	from fish sperm	Roche, Mannheim, Germany
Coomassie	Coomassie® Brilliant Blue G250	Fluka, Buchs, Switzerland
DNA ladder	GeneRuler™ 1kb DNA Ladder Plus	Fermentas, St.Leon-Rot, Germany
dNTPs	dATP, dCTP, dGTP, dTTP,	Roth, Karlsruhe, Germany
ECL	enhanced chemiluminescence (ECL)Western Blotting Substrate	Amersham Pharmacia, Freiburg, Germany
FOA	5-fluoroorotic acid	Melford Laboratories, Chelsworth, Ipswich, UK
milk	milk powder, blotting grade	Roth, Karlsruhe, Germany
Nitrocellulose membrane	Hybond ECL, nitrocellulose membrane	Amersham Pharmacia, Freiburg, Germany

Table 7. continued

Name	specification	Source
Pepton BactoTM	Peptone No. 3 Becton,	Dickinson and Company, Sparks, USA
Ponceau S	-	Serva, Heidelberg, Germany
Proteinase inhibitor	Complete, protease inhibitor cocktail EDTA-frei	Roche, Mannheim, Germany
Protein standard	Dual color precision plus protein standard	BIO-RAD, Hercules, USA
Silwet L-77	VAC-IN-STUFF (Silwet L-77)	Lehle Seeds, Round Rock,USA
TEMED	N, N, N', N'-Tetramethylethylendiamid	BIO-RAD, Hercules, USA
TRIS	UltraPureTM Tris Hydrochloride	Invitrogen, Karlsruhe, Germany
Triton X-100	pure	Serva, Heidelberg
Tween 20	Tween® 20	Sigma, Steinheim, Germany
Trypton BactoTM	Tryptone Becton	Becton, Dickinson and Company, Sparks, MD, USA
Yeast synthetic drop out medium	w/o H, T, L, U	Sigma, Steinheim, Germany
Yeast Nitrogen Base	Difco Yeast Nitrogen Base w/o amino acids	Becton, Dickinson and Company, Sparks, MD, USA
Other laboratory grade chemicals	.	Duchefa, Haarlem, Netherlands Fluka, Buchs, Switzerland Serva, Heidelberg, Germany Sigma, Steinheim, Germany Merck, Darmstadt, Germany Roth, Karlsruhe, Germany

II.1.9 Media

II.1.9.1 Bacterial growth medium

LB Luria Bertani Broth for *E.coli* cultivation

1% (w/v)	tryptone
0,5% (w/v)	yeast extract
0,5% (w/v)	NaCl

in dH_2O, autoclave for sterilization

For plates 1,5% (w/v) agar was added to the broth.

For selection kanamycin (50 µg/ml) or ampicilline (100 µg/ml) was added after autoclaving.

YEB medium for *A.tumefaciens* cultivation

0,5% (w/v) beef extract
0,1%(w/v) yeast extract
0,5% (w/v) tryptone
0,5% (w/v) sucrose
pH 7,2 adjusted with 0,5M NaOH

For plates 1,5% (w/v) agar was added to the liquid medium.
For selection carbenicillin (50 µg/ml), kanamycin (50 µg/ml) and rifampicin (50 µg/ml) was added after autoclaving.

II.1.9.2 Yeast cultivation medium

YEPD yeast full medium

20 g/l Difco peptone
10 g/l yeast extract
20 g/l glucose
in dH_2O, autoclave for sterilization
For plates 1,5% (w/v) agar was added to the liquid medium.

Yeast selective media

Components were mixed according to Table 8 (for drop out medium) and Table 9 (for absolute minimal medium) and autoclaved for sterilization. For plates 1,5% (w/v) agar was added to the liquid medium prior to autoclaving.

Table 8. Yeast drop out medium

Component per l	-H	-T	-HT	-HTU
Glucose (g)	20	20	20	20
Yeast nitrogen base (g)	6,7	6,7	6,7	6,7
Dropout mix selective aa (g)	-H/ 1,92	-T/ 1,92	-HTUL/ 1,4	-HTUL/ 1,4
Ura (mg)	-			
Leu (mg)	-		380	380
Agar	15	15	15	15
dH_2O			to 1l	

Table 9. Yeast absolute minimal medium

Component	-H	-T	-HT	-HTU
Glucose (g)	20	20	20	20
Yeast nitrogen base (g)	6,7	6,7	6,7	6,7
Ura (mg)	50	50	50	-
His (mg)	-	76	-	-
Trp (mg)	76	-	-	-
Lys (mg)	76	76	76	76
Leu (mg)	-	-	380	380
Agar	15	15	15	15
dH_2O			to 1l	

For FOA (5-fluoroorotic acid)-containing Minimal Medium (1L) 1g of FOA was dissolved in 10 ml of DMSO (or water) by vortexing and incubating at 37°C for 15-20 min. The FOA solution was added to the medium after autoclaving.

II.1.10 Buffers and solutions

II.1.10.1 Buffers for DNA work and cell biological experiments

Agarose gel

1 or 2%(w/v) Agarose

0,2 µg/l Ethidium bromide

in 1x TAE buffer

Aniline Blue staining solution

150 mM	KH_2PO_4
0,01% (w/v)	Aniline Blue
in dH_2O	
pH9,5 adjusted with KOH pellets	

Coomassie staining solution, 0,25%

Coomassie Blue 0,25% (w/v) in ethanol

$CaCl_2$- Solution for preparation of chemically competent *E.coli*

60 mM	$CaCl_2$
15% (v/v)	glycerol
10 mM	PIPES , pH 7,0

Autoclave for sterilization.

5 x DNA loading dye

50% (v/v)	glycerol
0,1% (w/v)	xylene cyanol
0,1% (w/v)	bromphenol blue
in dH_2O	

Edwards buffer for isolation of genomic DNA from plant material

200 mM	Tris/HCl pH7,5
250 mM	NaCl
25 mM	EDTA
0,5% (w/v)	SDS
in dH_2O	

PEG Solution for yeast transformation

in 1ml:

50% PEG 3350	680 µl
1M LiAc	100 µl
2mg/ml carrier DNA*	140 µl
dH_2O	80 µl

* The carrier DNA (10 mg/ml stock) was diluted (1:5) and incubated at 95°C for 5 min prior to use.

STETL buffer for *E.coli* boiling preparation

8% (w/v)	glucose
5% (v/v)	Triton X-100
50 mM	EDTA-Na_2-salt
50 mM	Tris pH8,0

in dH_2O

0,5 mg/ml Lysozyme was added prior to use

10 x TAE (Tris/acetate/EDTA) buffer for DNA gel electrophoresis

0,4 M	Tris
0,01 M	EDTA-Na_2-salt
0,2 M	acetic acid

in dH_2O

II.1.10.2 Buffers for protein work

Milk for blocking of membranes

5% (w/v)	milk powder

in 1 x TBS-T

10 x PBS (phosphate buffered saline)

0,038 M	$NaH_2PO_4(H_2O)$
0,162 M	Na_2HPO_4
1,5 M	NaCl

in dH_2O

Ponceau S staining solution

0,5% (w/v)	Ponceau S
5% (v/v)	acetic acid
in dH_2O	

Protein lysis buffer

10% (v/v)	glycerol
1% (v/v)	Triton X 100
5 mM	ß-mercapto ethanol
1 x complete mini protease inhibitor (Roche, Mannheim, Germany)	
in 1xPBS	

2 x SDS loading buffer (Laemmli buffer)

125 mM	Tris-HCl pH 6.8
20% (v/v)	Glycerol
2,5% (p/v)	SDS
0,0025% (p/v)	Bromophenol Blue
in dH_2O	
200mM DTT was added prior to use	

12% SDS-Polyacrylamide gels

The components listed in Table 10 were mixed and used to prepare 1,5mm polyacrylamide gels (see below).

Table 10. 12% SDS-PAGE

Component	12% separating gel (15ml)	Stacking gel (10ml)
dH_2O (ml)	3	3,4
Acrylamide 30% (ml)	6	830
1M Tris pH6,8 (µl)	-	630
1M Tris pH8,8 8 (ml)	5,7	-
10%APS (µl)	150	50
TEMED (µl)	6	5

10 x SDS-Running Buffer

250mM	Tris/HCl
2,5M	glycine
1%	SDS
in dH_2O	

10 x TBS (Tris buffered saline)

1,25M	NaCl
250mM	Tris/HCl pH 8,0
in dH_2O	

For 1xTBS-T 0,05% Tween-20 was added to the ten-times dilution.

Transfer Buffer

0,1% (w/v)	SDS
20% (v/v)	methanol
in 1x SDS-Running Buffer	

II.1.11 Plasmid vectors

A *PEN1* cDNA containing pDONR (S.Bau, personal communication; Invitrogen, Karlsruhe, Germany) was used as a template to generate site directed mutant variants of *PEN1* (see section II.2.7).

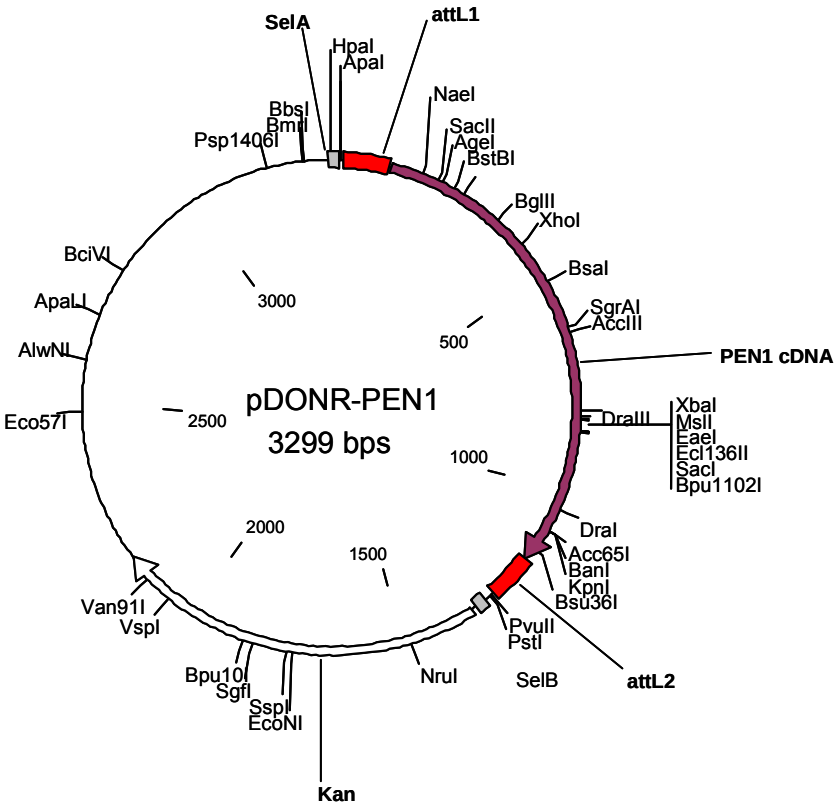


Figure 3. Map of the pDONR-PEN1 (S. Bau, personal communication). Sites of single cutting restriction enzymes are indicated. In red, attachment sites for LR reaction attl1 and attl2. Dark red arrow, PEN1 cDNA. White arrow, kanamycin resitance gene.

The GW-compatibel pAM-PAT obtained from B.Ülker was used to generate a binary transformation vector allowing the expression of fluorochrome-tagged PEN1 variants *in planta*.

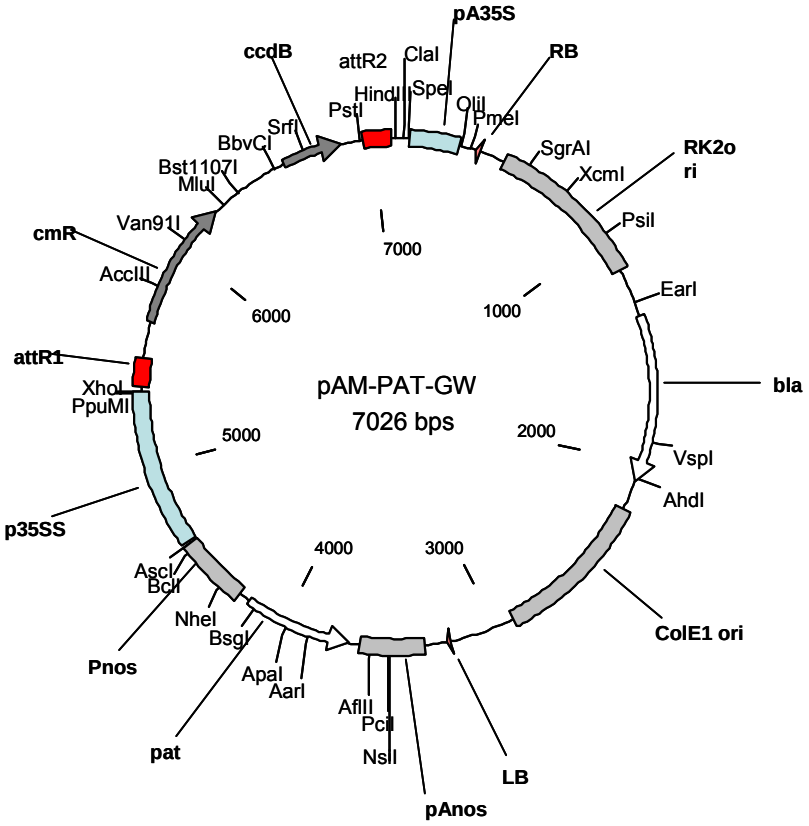


Figure 4. **Map of pAM-PAT** (B. Ülker, unpublished). p35S overexpression GW-compatible vector for agrobacterium-mediated transformation of *Arabidopsis*. Sites of single cutting restriction enzymes are indicated. In red, attachment sites attR1 and attR2 required for recombination with LR clonase. In grey, bacterial gene regulatory elements. In turquois, plant gene regulatory elements. White arrows, *pat* phosphoinothricin-acetyltransferase gene conferring BASTA resistance and *bla* ß-lactamase gene conferring ampicillin resistance. LB, left border; RB, right border; marking the DNA stretch transferred to the plant genome.

II.1.12 Molecular biological kits

Nucleo Spin Extract II kits (Macherery-Nagel, Düren, Germany) were utilized for PCR purification.

For plasmid preparation from *E.coli* cultures E.Z.N.A. Plasmid Mini Kit I (Omega-Biotek, Doraville, GA, USA) was used.

II.1.13 Microscopic equipment

A Zeiss Axiophot microscope (Carl Zeiss Jena, Jena, Germany) was used for bright field and epifluorescent imaging.

Confocal laser scanning microscopy was performed with a Leica TCS SP2 AOBS microscope (Leica Microsystems, Bensheim, Germany).

II.1.14 Software and internet resources

II.1.15 Statistical analysis

For statistical analyses SPSS software was used to run a Kolmogorov-Smirnov-Test to test for normal distribution of the data. Excel and was used to perform a two-sided heteroscedastic t-test to determine the statistical significance of the difference between two sample means, as listed in Table 11.

Table 11. Software

Program	Specification.	Source
Clone Manager 6	Cloning and sequence analyses software	SciEd Central, Cary, NC, USA
Chromas Version1.45	Sequence analysis	Conor McCarthy , Southport,Queensland, Australia
Excel 2003	Statistical analyses, t-test	Microsoft Cooperation, USA
SPSS 14.0 software	Statistical analyses Kolmogorov-Smirnov-Test	SPSS, Chicago, Illinois

II.1.16 Cloning and sequence analysis

Clone Manager was used for planning of cloning strategies, for the design of primers and the analysis of sequencing data (see Table 11). Sequence alignment was preformed using CustalW or Clone Manager. Annotated DNA sequences were obtained from online genome databases listed below in Table 12.

Table 12. Web resources

Database	Specification.	Web page
NCBI	National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
ClustalW	Sequence alignment	http://www.ebi.ac.uk/clustalw/index.html
TAIR	The Arabidopsis information resource	http://www.arabidopsis.org/
TIGR Rice v4.0	The institute for genomic research rice genome annotation	http://www.tigr.org/tdb/e2k1/osa1/
Poplar v1.0	Poplar genome annotation	http://genome.jgi-psf.org/Poptr1/Poptr1.home.html
ATTED-II	*Arabidopsis thaliana* trans-factor and cis-element prediction database	http://www.atted.bio.titech.ac.jp/
iHOP	Information hyperlinked over proteins	http://www.ihop-net.org/UniPub/iHOP/

II.2 Methods

II.2.1 Plant and pathogen cultivation

II.2.1.1 Cultivation of *A.thaliana*

A.thaliana seeds were sown on turf substrate (Stender Substrate, Wesel-Scharmbeck, Germany) including 0,001% Confidor WG70 (Bayer, Leverkusen, Germany) for protection against white flies. Seeds were stratified for two to three days at 4°C in complete darkness. To induce germination and allow for further under protected conditions the seeds were transferred to a Voetsch growth chamber with a 12 h light period and 60% humidity. The temperature was regulated to 22°C during light period and 20°C during darkness.

II.2.1.2 Cultivation of barley powdery mildew

Barley powdery mildew (*Blumeria graminis* formae specialae. *hordei* isolate K1) was propagated on *Hordeum vulgare* cultivar Ingrid 10, at 20°C, 70% relative humidity and a photoperiod of 16h light in a growth chamber.

II.2.1.3 Spore inoculation procedure

Plants were grown in a Voetsch growth chamber in protected pathogen-free conditions for three to four weeks, until the third true leaf pair was developed. Conidiospores of *B. g. hordei* colonies maintained on barley host plants were transferred to *Arabidopsis* plants by gentle shaking. The inoculation procedure was preformed using a settling tower to obtain an even distribution of the spores on the leaf surface of the test plants.
72 hours post inoculation (hpi) samples of the challenged leaves were taken. Three leaves (from the second and third true leaf pair) per plant were collected for counting of fungal entry rates, in parallel one to two residual leaves were cut from the same plants for immunoblot analysis. Per genotype three to four plants were sampled.
Leaves intended for assessing fungal entry rates, were destained in 70% ethanol by incubation at 65°C for 30min to several hours. Destained leaves were incubated overnight in aniline blue staining solution (see buffers and solutions) for visualizing callose deposition. To visualize extracellular fungal structures, i.e. spores, leaves were dipped in 0,25% Coomassie Blue staining solution, rinsed in water twice and mounted on microscopic slides. Fungal entry was scored using GFP-filter settings at a Zeiss Axiophot instrument (see II.1.13, microscopic equipment).

II.2.2 Molecular biology and cloning methods

II.2.2.1 Preparation of chemically competent *E.coli*

Ten ml of an overnight culture of *E.coli* DH5α was used to inoculate 800 ml of LB medium. Bacteria were grown at 37°C with shaking (225 rpm) for several hours to an OD_{590} of 0,375 and aliquoted 8 x 50 ml to pre-chilled, sterile Falcon tubes. The bacterial solution was left on ice for 10 min and then pelleted by centrifugation at 4°C, 7 min 3.000 rpm. The pellets were resuspended in 10ml cold $CaCl_2$ solution and re-pelleted by centrifugation at 4°C, 5 min, 2.800 rpm. The pellets were resuspended in 10 ml cold $CaCl_2$ solution and kept on ice for 30 min before another centrifugation at 4°C, 5 min, 2.800 rpm. Each pellet was resuspended in 2 ml of ice-cold $CaCl_2$ solution and the cells were aliquoted in 100 µl and stored at -80°C.

II.2.2.2 Transformation of chemically competent *E.coli*

An aliquot (100 µl) of chemically competent *E.coli* cells was thawed on ice.1µg of plasmid DNA was added to the cells. The cells were incubated on ice for 15 min and then heat-shocked at 42°C for 90 s and put an ice again for 15 min. 1 ml of LB medium was added and cells were incubated for 1 hr at 37°C, 225 rpm. 50 µl of the cells were plated on selective agar plates. The plates were incubated at 37°C overnight.

II.2.2.3 Preparation of electro-competent *Agrobacteria*

To prepare electro-competent *Agrobacteria* 10 ml YEB– medium containing the appropriate antibiotics were incubated with a single *Agrobacterium* colony and incubated overnight up to 2 days at 28°C with shaking, 200 rpm. Five ml of this culture was used as a starter to inoculate 500 ml YEB lacking antibiotics. The cells were incubated for 4 to 5 hours at 28°C with shaking at 200 rpm. The OD_{550} was adjusted to 0,3-0,5 with sterile water and transferred into chilled (4°C) Falcon tubes. Cells were centrifuged at 4°C for 10 min at 4.000 g. The resulting bacterial pellets were resuspended in 25 ml 1 mM HEPES (pH7,5). Again the cells were centrifuged at 4°C for 10 min at 4.000g. The cells were resuspended in 400 µl 1 mM HEPES containing 10% glycerol and frozen in 50 µl aliquots at – 80°C for long-term storage.

II.2.2.4 Transformation of electro-competent *Agrobacteria*

For transformation of *Agrobacteria*, 2mm electroporation cuvettes (Bio-Rad, München,Germany) were chilled on ice. Electro-competent *Agrobacteria* (50µl aliquots per reaction) were thawed on ice and 1 µg DNA was added to the cells and mixed by flipping the tube. The mixture was transferred to the pre-chilled cuvette prior to electro-pulsing using a Bio-Rad Gene Pulser apparatus at the following settings:

Gene Pulser	2,5 ∑V
Pulse Control	25 µF
Capitance Enhancer	400 Ω

One ml of YEB medium was added immediately after pulsing. The cells were transferred to a fresh Eppendorf tube and incubated for 2 h at 28°C. 100 µl of the cells were plated on YEB plates containing the appropriate antibiotics and incubated for 2 days at 28°C. Positive colonies were selected by PCR and for stable transformation of *Arabidopsis* plants.

II.2.2.5 Arabidopsis transformation (floral dipping method)

To generate stable transgenic line of *Arabidopsis thaliana* plants a genetic construct is integrated into the plant genome by an *Agrobacterium* mediated transformation (Shaw et al. 1983). The floral dipping method is widely used to transform flowering plants (modified from Clough and Bent 1998). The following protocol was used to prepare an *Agrobacterium* solution required for transformation:
400ml of YEB medium containing the appropriate antibiotics were inoculated with a 3 ml of pre-cultured *Agrobacteria* carrying a binary plant transformation vector; e.g. pPEN1::mYFP-PEN1 (see II.2.14). The culture was incubated for tow days at 28°C with shaking, 200 rpm. Cells were pelleted by centrifugation in a Kontron centrifuge at 4.800 rpm, 20 min, room temperature, Rotor A6.9. The pellet was resuspended in 150 ml 5% sucrose solution. The suspension was transferred into 1 l flasks and adjusted to OD_{600} 0,8 with 5% sucrose. *Arabidopsis pen1*-1 plants with several open inflorescences were used for floral dipping. Therefore the prepared *Agrobacterium* suspension was mixed with 100 µl Silwet-L-77 and transferred to a beaker. The *Arabidopsis* plants were inverted to plunge the inflorescences into the *Agrobacterium* suspension. The inflorescences were incubated for 30 s; soaking of the rosette leaves was avoided. After dipping the plants were kept under a cover with high humidity in dim light conditions for 18 to 24 h. Then the plants were transferred to the green hose and cultivated to set seeds.

II.2.2.6 BASTA selection of transformed *Arabidopsis* plants

Arabidopsis plants successfully transformed with a pAM-PAT derived binary vector carry the *pat* selective marker gene encoding for the enzyme phosphinothricin-acetyltransferase (PAT) which leads to increased tolerance to glufosinate-containing herbicides including BASTA (Bayer, Leverkusen, Germany). Seeds harvested from

transformed plants were sown on turf substrate in cultivation trays. After germination, the seedlings were sprayed with BASTA containing 18% (w/v) ammonium glufosinate. Survivors were tested for expression of the fluorescent fusion protein by fluorescence microscopy and immunoblot analysis.
The presence of the mutations in the *PEN1*-cDNA was confirmed by resequencing the transgene from genomic DNA isolated from plants in the T_2 generation.

II.2.3 Yeast transformation and split-ubiquitin two-hybrid assay

10ml o/n culture of yeast strain expressing the bait construct was grown in selective dropout media (-H). Cells were centrifuged for 5 min, 3000 rpm, at room temperature and resuspended in 10ml dH_2O. Cell suspension was centrifuged for 5 min, 3000 rpm at room temperature. The pellet was resuspended in 1ml 100 mM LiAc freshly prepared from a 1 M LiAc stock solution. The solution was transferred to a fresh Eppendorf tube and centrifuged for 15 s at 13000 rpm, room temperature. The pellet was resuspended in 0,5 ml 100 mM LiAc and cells were aliquoted in 50 µl in a 2 ml Eppendorf tube for each transformation. Then the yeast cells were pelleted by short centrifugation at 11000rpm, room temperature. 20 ng of plasmid DNA was added to the pellet. While vortexing the pellet, 300 ml of PEG solution was added and cells were incubated at 30°C for 30 min. Then cells were heat-shocked at 42°C for 60 min and afterwards the cells were centrifuged for 1 min at 13000 rpm, room temperature. The pellet was resuspended in 400 µl sterilized water. 10 µl was plated on selective media (-H-T). Colonies were picked after two to three days into 2 ml of liquid -H -T media. Serial dilutions were plated on FOA-containing selective -H -T media.

II.2.4 Plasmid preparation from *E. coli* (boiling preparation)

For fast preparation of plasmid DNA from *E. coli* boiling preparations were preformed. *E. coli* cells were grown over night in 3 ml of LB growth medium containing the correct antibiotics. 1,5 ml of the culture was transferred into a 1,5 ml Eppendorf tube and centrifuged in table top centrifuge at 15.000 g for 1 min at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µl STETL buffer. The resuspended cells were boiled for 30 s and then centrifuged again for 10 min, 15.000 g at room temperature. The amorphous pellet was removed using a sterile tooth pick and

100 µl isopropanol was added. The tube was inverted several times to gently mix the liquid. To pellet the plasmid DNA, the samples were centrifuges for 10 min, 15.000 g at room temperature. The supernatant was discarded and the pellet was resuspended in 50 µl H_2O containing 0,5 mg/ml RNase.

Sequencing-grade plasmid preparations were done using the E.Z.N.A. mini kit (Omega-Biotek, Doraville, GA, USA).

II.2.5 Isolation of genomic DNA from *Arabidopsis* leaf material

A protocol modified after Edwards et al. was used for preparation of genomic DNA from *Arabidopsis* leaf material (Edwards et al. 1991). One to two young and green leaves (1 cm^2/plant) were harvested in an 1,5 ml Eppendorf tube and frozen in liquid nitrogen. The tissue was grinded while still frozen and 400 µl Edwards buffer was added. The samples were incubated in a 65°C water bath for 30 to 60 min. To pellet the cell debris the samples were centrifuged for 5 min at 15.000 g at room temperature. Three hundred µl of the supernatant were transferred to a fresh 1,5 ml Eppendorf tube and 300 µl isopropanol was added. The liquid was mixed and the tubes were stored on ice for 30 min. To pellet the DNA the samples were centrifuged for 15 min at 15.000 g at room temperature and the supernatant was discarded. The pellets were washed with 70% ethanol and air dried before resuspending in 50 µl sterile water.

II.2.6 General PCR (polymerase chain reaction) protocol

Table 13. PCR conditions

component	final concentration.	µl per reaction
polymerase 1u/µl	0,5-1 u per reaction	0,5-1
forward primer (10µM)	200nM	1
reverse primer (10µM)	200nM	1
10xbuffer	1x	5
dNTPs (10µM)	200nM	1
template	-	1
dH_2O, sterile		20,5-21
total volume µl		50

PCR reactions were preformed in PCR-soft strip tubes, 0,2ml (Biozym Scientific, Hess.Oldendorf, Germany) using a PTC-225 Peltier Thermal Cylcer (MJ Research, Ramsey, MN, USA).

Table 14. PCR cycling

temperature	time	step	
94°C	3-10 min	according to GC content of template	
94°C	30 s		25 to 35 cycles
55-60°C	30 s	depending on primer melting temperature	
68-72°C	1-2 min per kb	temperature and time depending on polymerase	
68-72°C	5-15 min	depending on amplicon size	
4°C	15 min		

II.2.7 Site directed mutagenesis by splice-site overlap extension (SOE) PCR

To generate amino acid substitution variants of PEN1 splice-site extension PCR was preformed on a pDONR201 (Invitrogen, Karlsruhe Germany)-PEN1 template. Forward and reverse primers (see Table 6) carrying the desired mutations were designed (Table 6). Two PCR reactions each 25 cycles according to the general PCR protocol listed in Table 13 and Table 14 were preformed in parallel.

Table 15. SOE PCR conditions

component	final concentration.	µl per reaction
Pfu polymerase 1 u/µl	1 u per reaction	1
SeqL-A (10 µM)	200 nM	1
SeqL-B (10 µM)	200 nM	1
10xbuffer	1 x	5
dNTPs (10 µM)	200 nM	1
PCR product (*i*)	-	5-10
PCR product (*ii*)	-	5-10
dH_2O, sterile	-	19-29
total volume µl		50

Reaction (*i*) using the forward primer carrying the mutation and a reverse primer specific for the pDONR-PEN1 backbone (SeqL-B) see Table 6, and PCR (*ii*) using the reverse primer (complementary to the forward primer) carrying the mutation in combination with SeqL-A, annealing on the backbone of the pDONR in forward orientation. These reactions yield products overlapping in the site of the mutation. The products were purified and mixed to use as a template in a second SOE- PCR. Conditions and PCR cycle are indicated below in Table 15 and Table 16.The PCR products were column purified. The endogenous sequence in the pDONR-PEN1 backbone was exchanged by the recombinant PCR product using restriction digestion. The recombinant plasmid DNAs containing the predicted mutations were confirmed by DNA sequencing (see section II.2.15). The respective *PEN1* cDNAs were introduced by GATEWAY cloning technology (Invitrogen, Karlsruhe, Germany, see section II.2.10 below) into a binary plant transformation vector which I generated for these purposes, as described in section II.2.14.

Table 16. SOE-PCR cycle

temperature	time	step
94°C	5-10 min	add polymerase afterwards
94°C	30 s	
55°C	30 s	5 cycles to allow "template priming"
68°C	2 min 20 s	add SeqL-A/ SeqL-B afterwards
94°C	30 s	
55°C	30 s	20 cycles
68°C	2 min 20 s	
68°C	5 min	
4°C	15 min	

II.2.8 Purification of PCR products

PCR products were purified using Nucleo Spin Extract II kits (Macherery-Nagel, Düren, Germany).

II.2.9 Ligation

Ligation reactions were set up in 0,2ml PCR tubes and incubated at 16°C over night according to table Table 17.

Table 17. Ligation conditions

component	final concentration.	µl per reaction
T4 DNA ligase	1 u per reaction	1
linearized vector	50-200 ng	1
insert	3:1 ratio insert/vector)	3-7
10 xbuffer	1x	1
dH_2O, sterile		0-4
total volume µl		10

II.2.10 Gateway (GW) cloning

To introduce *PEN1* cDNAs carrying site-directed mutations from a pDONR backbone into the binary plant transformation vector (see Figure 5) recombination was achieved using the LR recombination technology (GATEWAY cloning, Invitrogen, Karlsruhe, Germany). To perform LR recombination reactions, the instruction of the manufacturer were followed. The LR reaction was set up as listed in Table 18.

Table 18. LR reaction conditions

component	final concentration.	µl per reaction
pDONR plasmid DNA	80 to 150 ng	1
pAMPAT plasmid DNA	80 to 100 ng	1
5 x LR reaction buffer	1x	1
LR-Clonase	-	1
ddH2O, sterile	-	1
total volume µl		55

II.2.11 Digestion of DNA using restriction enzymes

Restriction enzymes were used according to the manufacturer's protocol. Reactions were set up as indicated in Table 19 and incubated for 1 to 6hrs at the optimal temperature specified by the manufacturer.

Table 19. DNA restiction conditions

component	final concentration.	µl per reaction
restriction enzyme	1 -5 u per reaction	1-5
plamsid DNA or purified PCR product	0,5-2 µg	5-25
BSA (100x)	0-1 x	0-0,5
10 x buffer	1 x	5
dH_2O, sterile		15-39
total volume µl		50

II.2.12 DNA gel electrophoresis

PCR products or restriction digested DNA fragments were analyzed by gel electrophoresis. The samples were mixed with 5x DNA loading dye prior to loading on 1 or 2% agarose gels. DNA gel electrophoresis was preformed in 1xTAE buffer containing chambers at max.150V.

II.2.13 Generation of a domain swap construct between SYP122 and PEN1

A chimeric construct encoding the first 175 amino acids of SYP122 and the C-terminal 171 amino acids of PEN1 was constructed. Utilizing a SgrAI restriction site present in the cDNAs of both genes and an ApaI site present in the 5'region upstream of the cDNA in the pDONR backbone, the 5' half of PEN1 was excluded from the pDONR-PEN1 vector (see Figure 3). This piece was exchanged by the corresponding 5' part of SYP122 derived from a pDONR-SYP122 vector. Prior to ligation, the fragments were purified using a PCR purification kit (Macherey-Nagel, Düren, Germany). The resulting plasmids were sequenced to assure in-frame ligation.

II.2.14 Generation of a GW-compatible mYFP or cCFP-containing plant transformation vector

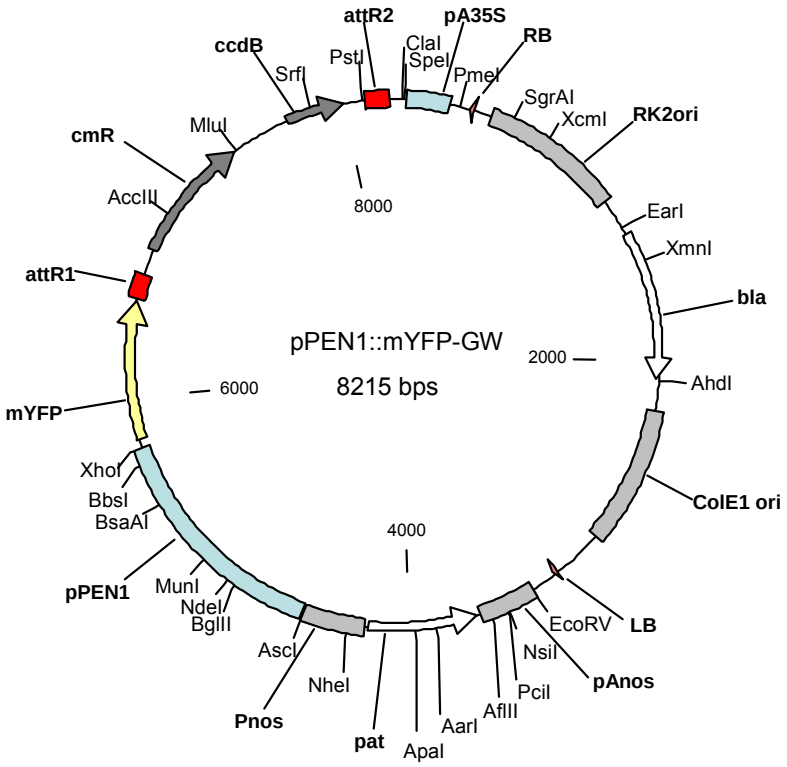


Figure 5. Map of the pPEN1::mYFP-GW. A pAM-PAT (C. Neu, B. Ülker, personal communication) derived GW-compatible vector for agrobacterium-mediated transformation of *Arabidopsis*. Single site cutting restriction enzymes are indicated. In red, attachment sites attR1 and attR2 required for recombination with LR clonase. In grey, bacterial gene regulatory elements. In turquois, plant gene regulatory elements including pPEN1. White arrows, *pat* phosphoinothricin-acetyltransferase gene conferring BASTA resistance and *bla* ß-lactamase gene conferring ampicillin resistance. Yellow arrow, mYFP cDNA. LB, left border; RB, right border; marking the DNA stretch transferred to the plant genome.

A Gateway (GW, Invitrogen, Karlsruhe, Germany)-compatible binary plant transformation vector, suitable for the expression of mYFP and cCFP (monomeric yellow fluorescent protein, and cerulean fluorescent protein; Zhang et al. 2002) -tagged gene of PEN1 variants, under the control of the 1,2kb of native upstream regulatory

PEN1 sequence (pPEN1, Collins et al. 2003) or under the 35S promoter (p35S) for strong expression (Paszkowski et al. 1984, Sanders et al. 1987) was generated on the base of a pAM-PAT vector (B.Ülker, unpublished). cDNA sequence encoding the fluorochrome (either mYFP or cCFP) was amplified using the primer pair spa098 /spa088 to introduce a 5' XhoI-AatII and 3' SalI site. The PCR-fragment was column-purified prior to digestion with XhoI and SalI. The fragment was ligated to the backbone of pAMPAt-GW opened with XhoI, yielding p35S::mYFP (or cCFP)-GW.
To exchange the 35S promoter to pPEN1, pPEN1 was amplified from plasmid DNA (S.Bau, unpublished) using spa074 and spa097 to introduce terminal AatII and AscI sites for cloning. AatII and AscI restriction was used to remove p35S and ligation of the pPEN1 PCR fragment resulted in pPEN1::mYFP-GW, as depicted in Figure 5.

II.2.15 DNA sequencing

DNA sequences were determined by the MPIZ DNA core facility on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377, 3100 and 3730 sequencers using BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems. Oligonucleotides were purchased from metabion (Martinsried, Germany), operon biotechnologies (Cologne, Germany) and Sigma Genosys (Steinheim, Germany).

II.3 Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed with a Leica TCS SP2 AOBS microscope equipped with an Argon/Helium-Neon laser and diode laser of 405 nm. Detached leaves of ten day old plantlings were mounted in water on microscopic slides for imaging. Excitation of the samples was preformed at 488 nm for GFP, at 514 nm for mYFP and 405 nm for cCFP. Emission spectra were taken at 491 to 551 nm for GFP, at 518 to 578 nm for mYFP, and 435 to 500 nm for cCFP. Aniline blue stained samples were excited using the 495 nm diode laser and the emission was taken at 410 to 480 nm. For propidium iodide stained *B.g. hordei* spores the excitation was set to 561 nm and fluorescence emission was measured at 600 to 705 nm. Images were processed using Adobe PHOTOSHOP 7.0 (Adobe Systems Inc., San Jose, CA,USA).

II.3.1 Plasmolysis

To monitor plasmolysis, ten day old *Arabidopsis* leaves expressing the respective fluorochrome-tagged SNARE protein were mounted on microscopic slides, flooded with 5 M sorbitol and imaged immediately. However, permeation of sorbitol into leaf epidermal cells was not always immediately effective and sometimes plasmolysis was delayed for several minutes.

II.3.2 Time lapse microscopy

For time lapse microscopic imaging the Leica TCS SP2 AOBS software tool was used. The imaging interval was minimized and images were taken every 5 to 15 s in a time interval of 60 to 180 s, depending on the line average scanning settings.

II.4 Protein biochemical methods

II.4.1 Immunoblot analyses

II.4.1.1 Protein extraction from plant leaf material

One to two frozen Arabidopsis (approximately 1 cm^2) leaves were grinded in liquid nitrogen. 150 µl protein lysis buffer (see buffers and solutions) was added and samples were kept on ice until last sample was prepared. The samples were vortexed thoroughly and centrifuged at 4°C at maximum speed for 15 min using a table top centrifuge. The supernatant was transferred to a new Eppendorf tube and samples were kept on ice. Protein concentration in each sample was determined using the Bradford reagent. For detection with PEN1-antiserum 5 µg of total protein extract was loaded onto a 12% SDS-PAGE (see below). For detection with SYP122-antiserum 20 to 30 µg of total extract was loaded.

II.4.1.2 Protein extraction from yeast cells

Transformed yeast cells were grown in 10 ml of selective medium o/n to an OD_{600} of 1,0 to 2,0. Two OD_{600} units were harvested in 2 ml Eppendorf tubes by centrifugation. The samples were washed in 250 µl dH_2O, and centrifuged again. The samples were frozen in liquid nitrogen and then boiled for 5min. This step was repeated three times. Then 200µl of 2x SDS loading buffer with 20% 0.2 M DTT was added. The samples were boiled again for 5 min and loaded on a 12% SDS-PAGE. For detection the HA antibody was used in 1:10000 dilution in 1 x TBS-T.

II.4.2 SDS-polyacrylamide gel electrophoresis (PAGE) and Western-blotting

To separate proteins under denaturing conditions according to their size, SDS-PAGE was performed using the discontinuous Laemmli procedure (Laemmli 1970). A stacking gel on top of the separating gel concentrates the proteins before they are separated in the lower separating gel. The negatively charged sodium dodecyl sulfate (SDS) molecule binds to most proteins in a constant ratio (1,4 g/g protein) resulting in negatively charged SDS-protein complexes, which can be separated according to their size only.
Protean 3 mini gels (1,5 mm; Bio-Rad, München, Germany) were used. To generate the polyacrylamide gels, the separating gel was poured and covered with a layer of isopropanol to ensure a homogenous gel margin. The stacking gel was poured on top of the polymerized separating gel after removal of the isopropanol. Protein samples were loaded including a protein standard (Precision Plus Protein Standard; Bio-Rad, München, Germany). The gels were run at 100 to 150V in 1x SDS-running buffer until the sample running front reached the gel bottom. Wet blotting of the gels onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia, Freiburg, Germany) was preformed overnight at 40 mA in Bio-Rad Mini-Transblot Electrophoretic cells according to Towbin et al (Towbin et al. 1979).

II.4.3 Immunodetection of proteins

Following the blotting procedure, the membranes were rinsed in water and incubated for 15min in Ponceau S staining solution. The staining of the membranes was imaged for

documentation. Destaining was achieved by washing the membranes twice in 1 x TBS-T for 5 min. The membranes were blocked in 5% milk for 1,5 to 2 h at room temperature. After the blocking step the membranes were washed three times for 5min in 1 x TBS-T and incubated with the primary antibody dilution for 1 h at room temperature. Again a washing step was included (three times, 5 min in 1 x TBS-T) before incubation with the secondary horse radish peroxidase-coupled antibody for 1 h at room temperature. The secondary antibody was decanted and the membranes were washed three times for 5 min in 1 x TBS-T. For detection the blots were incubated with chemi-luminescence detection solution (ECL, Amersham Pharmacia, Freiburg, Germany) and light emission was documented on x-ray films (Hyperfilm, Amersham Pharmacia, Freiburg, Germany).

III Results

III.1 Structure-function analysis of the PEN1 protein

To elucidate PEN1 structure-function relationships, I have generated a set of PEN1 amino acids substitution variants by site-directed mutagenesis (summarized in Figure 6 and Table 20).

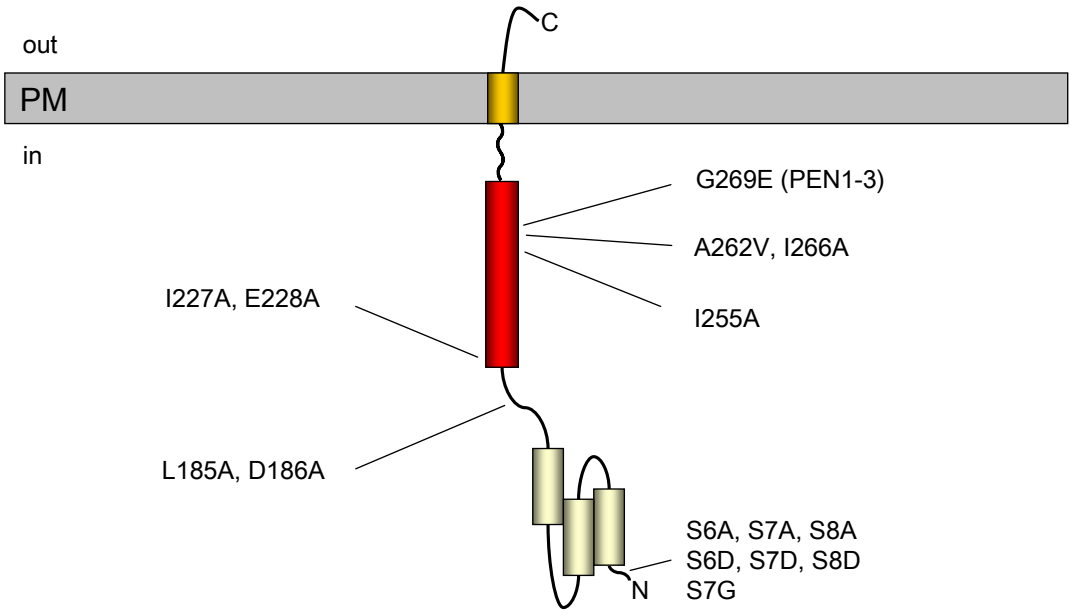


Figure 6. Schematic drawing depicting the PEN1 domain structure. Amino acid substitutions introduced into the PEN1 sequence are indicated. In orange, membrane-spanning helix; in red, Qa SNARE domain; in yellow, H_{abc} regulatory helices.

The respective amino acid substitutions were either chosen on the basis of conserved N-terminal phosphorylation sites shared between *Arabidopsis* SYP122 and PEN1 (Nuhse et al. 2003), or target conserved residues that were previously shown to be required for various functions of animal t-SNAREs (Dulubova et al. 1999, Dulubova et al. 2003, Fergestad et al. 2001, Richmond et al. 2001, Wu et al. 1999).

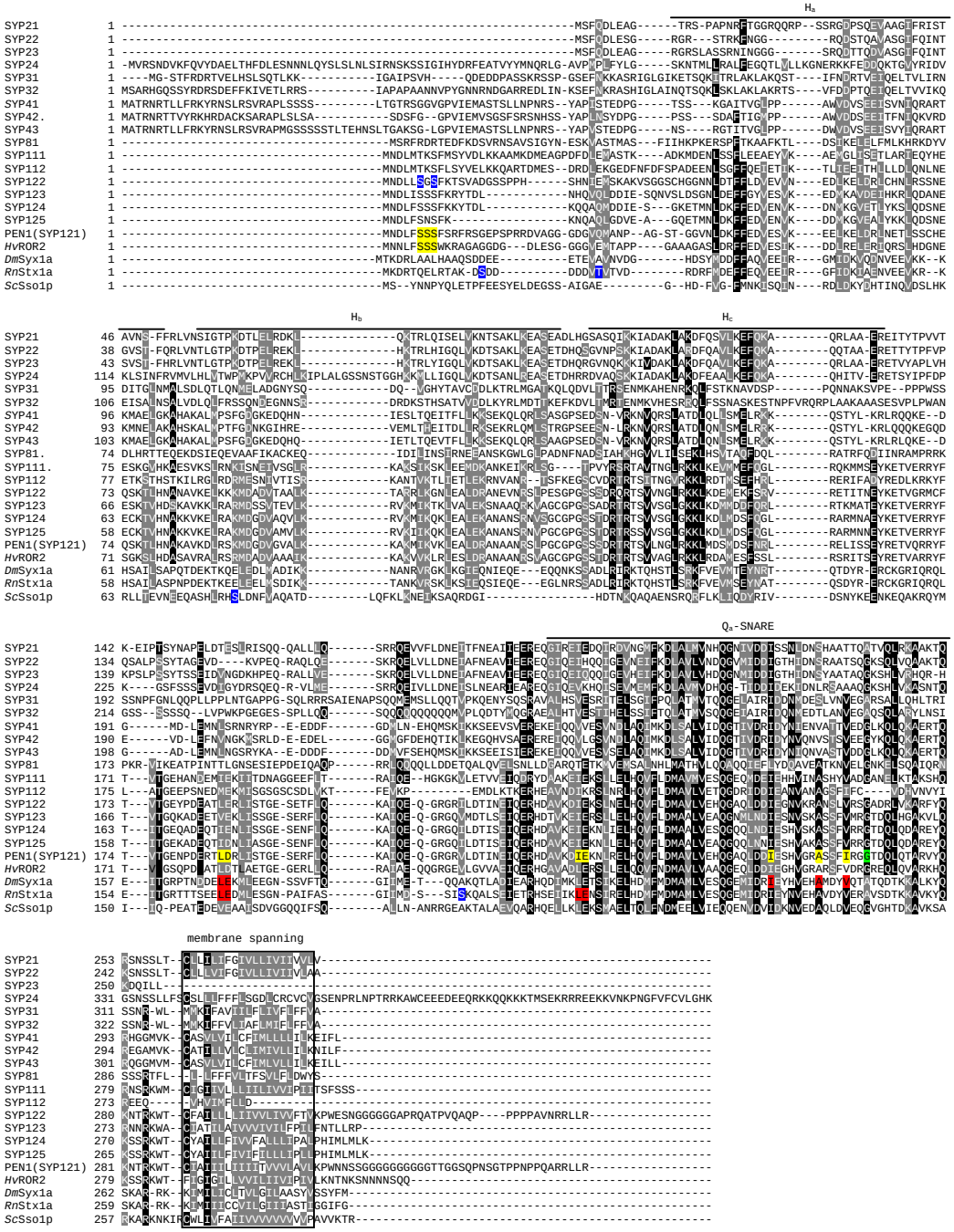


Figure 7. Amino acid sequence alignment of the *Arabidopsis* syntaxin family and PEN1-related syntaxins from *Hordeum vulgare*, *Drosophila melanogaster*, *Rattus norvegicus* and *Saccharomyces cerevisiae*. Amino acids of targeted by site-directed substitution and characterized implicated in syntaxin functions in animal systems (see Figure 6 and Table 20) are highlighted in red. Amino acids in PEN1 targeted for substitution are marked in yellow.

Figure 7 continued. Amino acids reported to be phosphorylated in *Arabidopsis* SYP122, rat RnStx1a or yeast SsSso1p are marked in blue and the PEN1-3 mutation (G269E) in green. Regions of sequence conservation are highlighted in black (identical amino acids) and in grey (similar amino acid). H_a, H_b, H_c, helices define deduced regulatory helical bundles of PEN1.

This includes residues involved in binding to Sec/Munc (SM)-like regulatory proteins in mammalian syntaxin (e.g. *Rattus norvegicus* syntaxin 1a; *Rn*Stx1a, see Figure 7) and *D. melanogaster* syntaxin (*Dm*Syx1a, see Figure 7; Dulubova et al. 1999, Wu et al. 1999), residues involved in binding to SNAP25 in mammalian syntaxin 1a (Dulubova et al. 1999), residues involved in ternary SNARE complex stability in mammalian and *D. melanogaster* syntaxin 1a (Bezprozvanny et al. 2000, Fergestad et al. 2001), and residues important for conformational changes between "open" and "closed" states in mammalian syntaxin 1a and *C. elegans* syntaxin *Unc-64* (Dulubova et al. 1999, Richmond et al. 2001). Amino acids substituted in PEN1 are highlighted in red color in the sequence alignment in Figure 7, which illustrates the extent of sequence conservation with other plant and animal syntaxins. The pictogram in Figure 6 indicates the amino acid substitutions with respect to the PEN1 domain structure as inferred from the known structure of syntaxin 1a (Sutton et al. 1998).

In total, I generated ten amino acid substitution variants of PEN1 by site-directed mutagenesis (summarized in Table 20 and depicted in Figure 6; see Materials and Methods II.2.7). The respective *PEN1* cDNAs were introduced into a binary plant transformation vector (pPEN1::mYFP-GW, p35S::mYFP-GW or p35S::cCFP-GW; see Materials and Methods II.2.14), which I generated for these purposes . To enable sub-cellular localization studies of the PEN1 variants by fluorescence microscopy a cDNA encoding the monomeric yellow fluorescent protein (*mYFP*)- or cerulean cyan fluorescent protein (*cCFP*)- marker was cloned into the vector resulting in N-terminally tagged fluorochrome-fusion proteins (see Materials and Methods II.2.14). The expression of the mYFP-fused transgene in *pen1*-1 mutant plants was driven by either 1,2kb of *PEN1* 5`regulatory sequence (designated here PEN1 promoter or pPEN1) for native, or the 35S cauliflower mosaic virus promoter (p35S) for overexpression, (see Materials and Methods II.2.14; Herrera-Estrella et al. 1983, Paszkowski et al. 1984, Sanders et al. 1987). Each of the engineered *PEN1* variants was subsequently

characterized *in planta* for their ability to mediate resistance responses (see Results section III.1.2 to III.1.4), their ability to accumulate underneath attempted fungal entry sites, and in yeast two-hybrid experiments for potentially altered protein-protein interactions with MLO2 (see Results III.2).

Table 20. Amino acid substitutions introduced into PEN1.

Amino acid exchanges introduced* in PEN1	Posttranslational modification in Syp122 or reported effects of substitutions in animal syntaxins	Reference
S6A	potentially phosphorylated in SYP122	Nühse et al 2003
S7A	potential phosphorylation motif	Nühse et al 2004
S8A	potentially phosphorylated in SYP122	Nühse et al 2003
S6A, S7A, S8A	potentially phosphorylated in SYP122	Nühse et al 2003
S6D, S7D, S8D	potentially phosphorylated in SYP122	Nühse et al 2003
S7G	potential phosphorylation motif	Nühse et al 2004
L185A, D186A	*Rn* Syntaxin 1a open conformation *in vitro*, rescues unc13-deficiency in *C. elegans*	Dulubova et al 1999, Richmond et al 2000
I227A, E228A	*Rn* Syntaxin1a disruption of SNAP25 binding *in vitro*	Dulubova et al 1999
I255A	*Dm* Syntaxin1a defective in Munc-18 binding *in vitro,* altered neurosecretion in flies *in vivo*	Wu et al 1999
A262V, I266A	*Rn* Syntaxin1a decreased SNARE complex stability *in vitro,* disturbed neurotransmission in transgenic *Drosophila* embryos.	Bezprozvanny et al 2000 Fergestad et al 2001

* according to PEN1 sequence (At3g11820)

III.1.1 pPEN1 confers wild-type-like protein levels of PEN1 variants in transgenic *Arabidopsis* lines.

Transgenic plants expressing wild-type-like levels of the tested PEN1-fusion proteins were selected by immunoblot analysis using a PEN1-specific antiserum (provided by H.T-Christensen). The expected molecular weight of fluorochrome-tagged PEN1 is 64 kD (37 kD PEN1 plus 27 kD GFP). Total protein extract from transgenic leaf

material probed with the PEN1-antiserum yielded two bands after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) based size separation

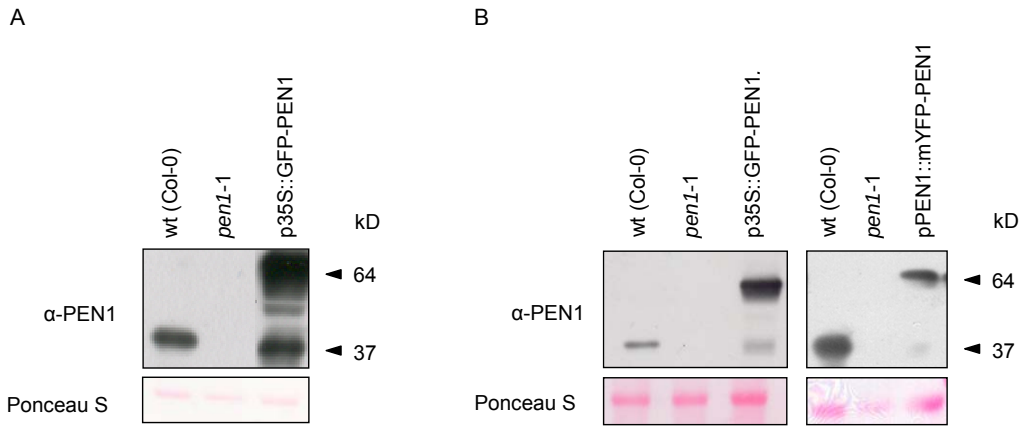


Figure 8. Cleavage of mYFP-PEN1 fusion protein is dependent on protein extraction conditions. Immunoblot analysis of leaf protein extract from total leaf material of wild-type, mutant, and transgenic plants. Total protein extract from leaf material of *pen1*-1 plants either expressing a pPEN1::mYFP-PEN1 or the p35S::GFP-PEN1 construct was separated on SDS PAGE and detected with PEN1 antiserum. (A) Protein extraction in protein lysis buffer (see Materials and Methods, II.1.10.2). (B) Protein extraction in 1x SDS loading buffer (see Materials and Methods, II.1.10.2). The 64 kD band represents the full-length fusion protein. The 37 kD signal is the expected size of un-tagged PEN1 protein. Ponceau S staining demonstrates equal loading of protein amount per lane. wt, wild type.

One band migrating at the expected size of the full length fusion protein of 64 kD, and a second signal at 37 kD, the predicted size of untagged PEN1 (Figure 8A). Assuming that the translation of the transgene mRNA is initiated at the correct ATG start codon upstream of the *mYFP* fluorochrome cDNA, the 37 kD band is might result from cleavage of the fusion protein potentially by an *Arabidopsis* protease released during the extraction procedure. The use of different protease inhibitors did not reduce the presumed proteolytic cleavage (data not shown). However, when the protein extraction was performed in the presence 1xSDS sample buffer instead of protein lysis buffer, the ratio of intact mYFP-PEN1 in comparison to "mYFP-cleaved" PEN1 increased substantially (Figure 8B). In native pPEN1 promoter lines, cleaved mYFP-PEN1 signal was undetectable following protein extraction in 1 x SDS sample buffer. This indicates that *in planta* the majority of the fusion protein is intact and that the detected cleavage

of the fusion protein is a result of the extraction procedure. Unfortunately, the improved extraction procedure was uncovered at a late time point of my PhD project. For this reason most immunoblots shown below were generated with protein samples obtained using the original protein lysis buffer.

III.1.2 mYFP-PEN1 rescues the *pen1*-1 fungal entry phenotype

To test whether the mYFP-PEN1 wild-type fusion protein was functional, complementation of the *pen1*-1 fungal entry phenotype was assessed by inoculation experiments of the transgenic lines with conidiospores of the barley powdery mildew *B. graminis* fsp *hordei* isolate K1 (designated *B. g. hordei* further on). Spores were inoculated on leaves of three to four week-old seedlings and fungal entry rates into leaf epidermal cells were determined microscopically at 72 hours post inoculation (hpi; Figure 9). Four independent transgenic lines of pPEN1::mYFP-PEN1 in *pen1*-1 and the overexpression line p35S::GFP-PEN1 in *pen1*-1 (Collins et al. 2003) were analyzed, see Figure 9. For statistical analysis, normal distribution of the two control data sets (entry rates in Col-0 and *pen1*-1) was assessed by performing a Kolmogorov-Smirnov test (Henderson 2006, Lilliefors 1967) and student's two-tailed t-test (Student 1908) was used for comparison of the data sets obtained.

The fusion protein was able to complement the *pen1*-1 null mutant phenotype to near Col-0 wild-type entry rates of 15 (+/-1) %. In the native promoter lines pPEN1::mYFP-PEN1 #2, (designated PEN1 #2 further on), and PEN1 #7, and in the overexpression line p35S::GFP-PEN1, *B. g. hordei* entry rates of 20 (+/-3) %, 21 (+/-2) %, and 24 (+/-3) % were detected. In contrast, in lines PEN1 #4 and #6 fungal entry rates were clearly elevated up to 30 (+/-5) % and 28 (+/-6) %, respectively, compared to Col-0 wild type (see Figure 9). Statistical analysis revealed no significant differences between transgenic lines PEN1 #2 and #6 and the wild-type control ($P = 0{,}18$ and $P = 0{,}11$, respectively) as indicated by the asterisks in Figure 9. The fusion construct was therefore considered to be functional in these lines.

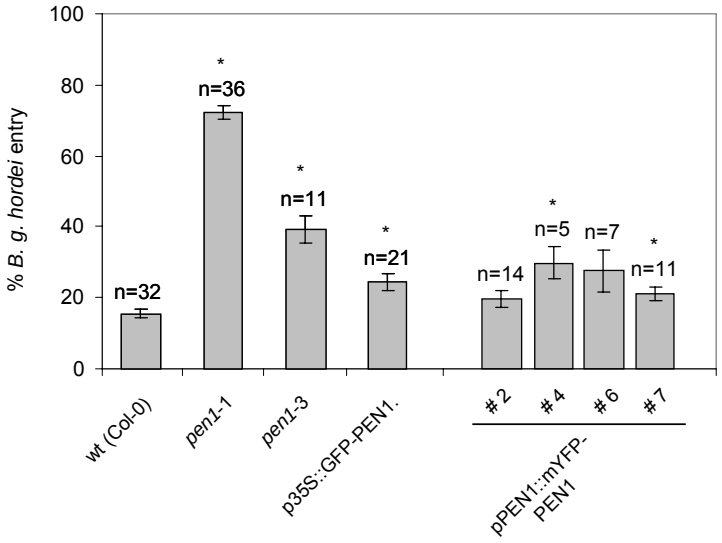


Figure 9. The mYFP-PEN1 fusion protein complements the *pen1*-1 mutant phenotype. Four independent transgenic lines expressing pPEN1::mYFP-PEN1 at wild-type levels were challenged with *B. g. hordei* spores. Fungal entry rates were analyzed 72 hours post inoculation (hpi). Figure columns represent at least 5 individuals (n) from at least two independent biological replicates. Results obtained from plants of the T_2 and T_3 progeny of identical T_1 transformants were pooled. *pen1*-1 is a predicted null mutant, *pen1*-3 is a ethyl methane sulfonate (EMS)-derived partially non-functional allele of *PEN1*. Error bars represent the SEM; n, number of individuals scored; *, statistically significant difference in comparison to Col-0 wild-type control (P > 0,05). #2, #4, #6, #7 indicates independent transgenic lines. n, number of individual plants tested.

The observed variation of fungal entry rates ranging from 20 to 30% between individual transgenic lines was not correlated with differences in fusion protein abundance at 72 hpi. However, samples were not taken before fungal challenge and thus it remains possible that differences in steady state levels of the fusion protein in individual transgenic lines account for the detected variation. Alternatively, transgene copy number, transgene position in the genome, and homo/hemizygosity of the transgene might explain the observed quantitative variation of complementation in the individual lines.

Although the slight increase in entry fungal rates of complementation line PEN1 #2, in comparison to Col-0 wild-type plants was statistically not significant (P = 0,176), a minor inhibitory effect of the N-terminally fused mYFP-fluorochrome cannot be

excluded. Syntaxin-like t-SNAREs undergo extensive structural changes upon binary and ternary complex formation involving an α-helical bundle close to their N-terminus (Bracher and Weissenhorn 2004, Dulubova et al. 1999, Margittai et al. 2003). These conformational have been shown to be essential for syntaxin function in *C. elegans* (Richmond et al. 2001). It is conceivable that such essential changes could be affected by the rather bulky fluorochrome tag.

III.1.3 Phosphorylation at N-terminal residues may contribute to PEN1 function

Phosphorylation and dephosphorylation play important roles in the regulation of SNARE protein function in yeasts and animals (Gerst 2003, Gurunathan et al. 2002, Marash and Gerst 2003, Nagy et al. 2004, for review see Snyder et al. 2006, and Turner et al. 1999). In plants, SNARE protein phosphorylation has not been thoroughly studied. Interestingly, two studies using cultured *Arabidopsis* cells report phosphorylation of N-terminal syntaxin residues in SYP122 and PEN1 upon treatment with the flg22-peptide derived from bacterial flagellin (Benschop et al. 2007, Nuhse et al. 2003). The flg22-peptide is a well-characterized pathogen-associated molecular pattern (PAMP) that induces PAMP-triggered plant immune responses via the FLS2 PAMP-receptor (Felix et al. 1999, Gomez-Gomez et al. 1999, Zipfel et al. 2004). Similarly, the proposed tobacco ortholog of PEN1, *Nt*SYP121 was shown to be phosphorylated during *R*-gene triggered resistance responses (Heese et al. 2005). The N-terminal serine residues reported to be phosphorylated in SYP122 and PEN1 are conserved in *Nt*SYP121. However, the phosphorylation sites in *Nt*SYP121 have not been determined in this previous study (Heese et al. 2005).

To address the question whether N-terminal serine phosphorylation in PEN1 may play a role in disease resistance to non-adapted powdery mildew fungi, three PEN1 serine exchanges variants of PEN1, PEN1^{S7G}, PEN1$^{S6A, S7A, S8A}$, and PEN1$^{S6D, S7D, S8D}$ were generated and analyzed for their ability to mediate disease resistance responses in transgenic *Arabidopsis* lines expressing these PEN1 variants (Figure 10). For this purpose, fungal entry rates were determined in the transgenic lines upon spore inoculation with *B. g. hordei*.

PEN1^{S7G} variants showed a small increase in fungal entry rates compared to the wild-type transgene (PEN1#2). This increase was statistically significant for lines PEN1^{S7G} #2 and #4 (P = 0,049 and P = 0,025), but not for line PEN1^{S7G} #1 (P = 0,191).

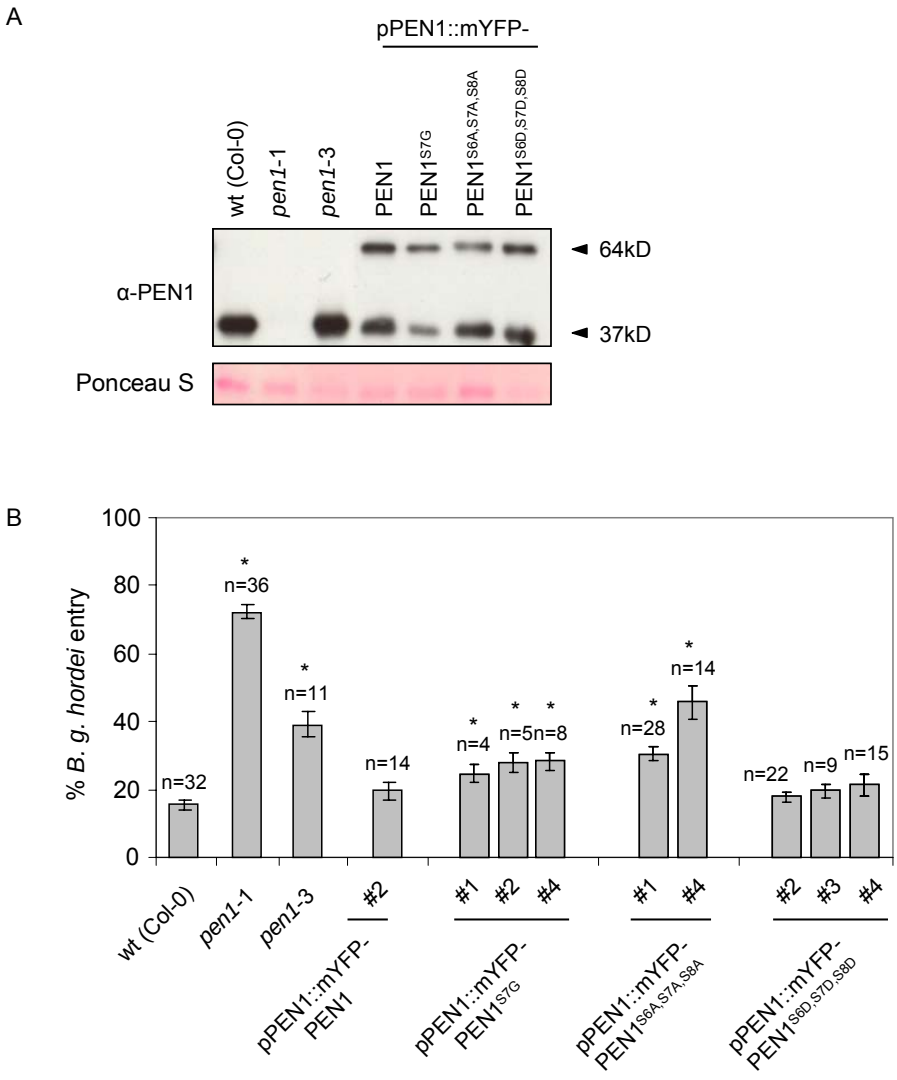


Figure 10. N-terminal phosphorylation but no de-phosphorylation appears to be required for full activity of PEN1. (A) Immunoblot analysis probed with PEN1-antiserum. One independent transgenic line per construct representing typical protein levels is depicted in this experiment. (B) *B. g. hordei* entry rates into leaf epidermal cells at 72 hpi. Figure columns represent at least 4 individuals (n) from at least two independent experimental replicates. Results obtained from plants of the T_2 and T_3 progeny of T_1 transformants were pooled. Error bar represents SEM; n, number of individuals scored; *, statistically significant difference to Col-0 control ($P > 0,05$).

Thus, a substitution of serine 7 with glycine in PEN1 had minor effects on PEN1 activity in non-host resistance to *B. g. hordei.* In contrast, two tested lines in which all three N-terminal serine residues are rendered inaccessible to phosphorylation or de-

phosphorylation PEN1$^{S6A, S7A, S8A}$ (lines #1 and #4) showed enhanced rates of fungal ingress of 30 (+/-2) % and 46 (+/-5) %, respectively (Figure 10), which differed significantly from the control lines PEN1 #2 (P = 0,003 and P = 0,0002) and Col-0 wild type (P = 2,96 E^{-07} and P = 3,44 E^{-05}). Indeed, fungal entry rates in PEN1$^{S6A, S7A, S8A}$ lines were in the range of the partially defective ethyl methane sulfonate (EMS)-derived mutant *pen1*-3, which shows 39 (+/-4) % of *B. g. hordei* ingress. Interestingly, each of three tested PEN1$^{S6D, S7D, S8D}$ transgenic lines complemented the *pen1*-1 phenotype to *B. g. hordei* at levels comparable to the wild-type control construct PEN1 #2 and to Col-0 wild-type plants (P = 0,292 for PEN1$^{S6D, S7D, S8D}$ #2; P = 0,215 for PEN1$^{S6D, S7D, S8D}$ #3; and P = 0,098 for PEN1$^{S6D, S7D, S8D}$ #4). Thus, the activity of the presumed phospho-knockout variant of PEN1$^{S6A, S7A, S8A}$ was reduced, but not the activity of the putative phospho-mimic for PEN1$^{S6D, S7D, S8D}$. Assuming that the introduced substitutions do not cause considerable conformational changes in PEN1, these data support a potential functional contribution of syntaxin phosphorylation in non-host resistance to powdery mildew pathogens. Specifically the finding might suggest that the phosphorylation of N-terminal serine residues is required for full activity whilst de-phosphorylation might not be essential.

III.1.4 Amino acid exchanges in and adjacent to the SNARE domain alter PEN1 function

In animal syntaxins, including *C. elegans* syntaxin 1, *R. norvegicus* syntaxin 1a and *D. melanogaster* syntaxin 1a the effect of mutations of conserved amino acids in the SNARE domain and the linker region connecting the N-terminal regulatory H_{abc} helices to the SNARE domain have been studied *in vitro* and *in vivo* (see Introduction section I.2; Bezprozvanny et al. 2000, Dulubova et al. 1999, Fergestad et al. 2001, Richmond et al. 2001, Wu et al. 1999).

In this study, I introduced some of the previously characterized mutations of animal syntaxins into the PEN1 sequence to assess their potential contribution to PEN1 activity in disease resistance responses. Transgenic lines of each PEN1 derivative whose protein levels were similar to the Col-0 wild-type plants were chosen for subsequent inoculation experiments (Figure 11A). Plants were grown as described above (see chapter II.2.1.1) and subjected to *B. g. hordei* spore inoculation (Figure 11B, and II.2.1.3).

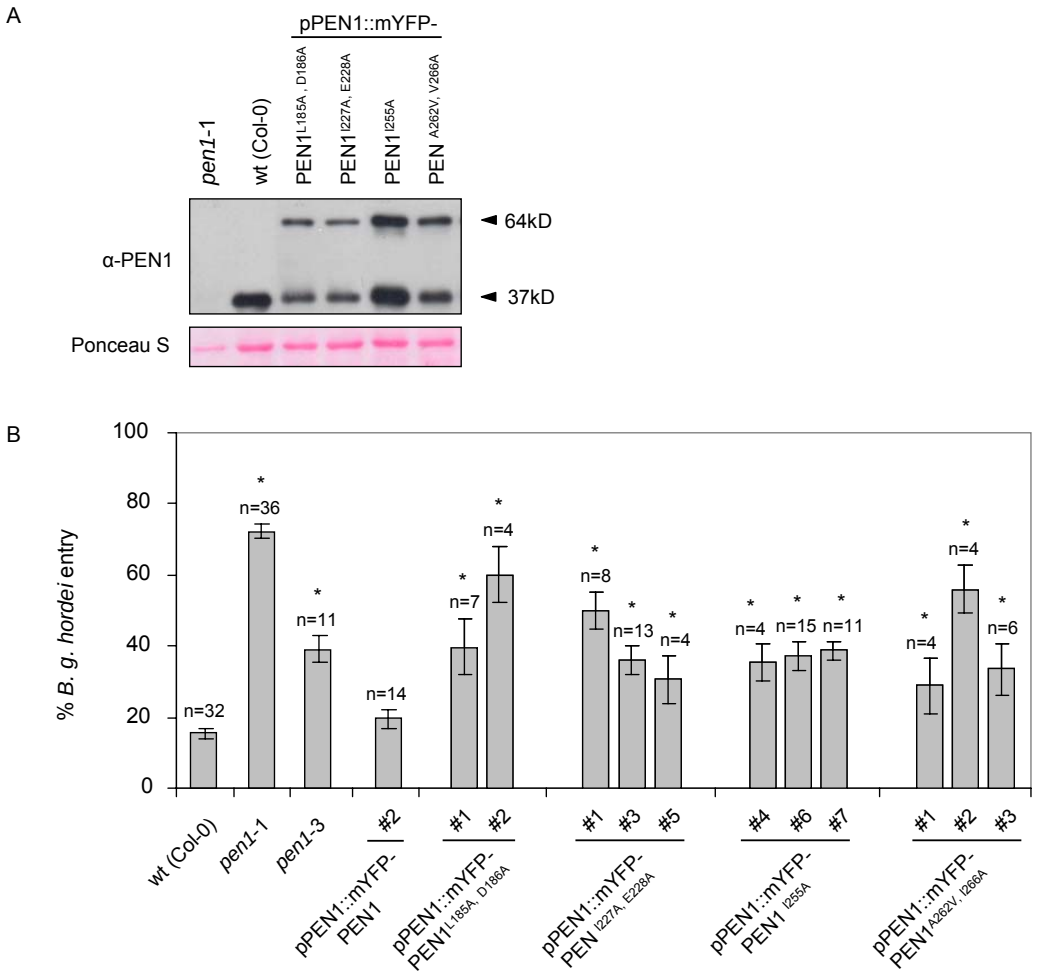


Figure 11. Amino acid exchanges in the SNARE domain and the linker region between the SNARE domain and the regulatory H_{abc} helices of PEN1 reduce PEN1 activity in disease resistance. (A) Immunoblot analysis probed with PEN1-antiserum. One independent transgenic line per construct representing typical protein levels is depicted in this experiment. (B) Fungal entry into leaf epidermal cells at 72 hpi. Figure columns represent at least 4 individuals (n) from at least two independent experimental replicates. Results obtained from plants of the T_2 and T_3 progeny of T_1 transformants were pooled. Error bars represent the SEM; n, number of individuals scored; *, statistically significant difference to Col-0 control (P > 0,05).

All amino acid exchanges introduced in the SNARE domain or in the linker region between the regulatory helices and the SNARE domain reduced PEN1 activity in disease resistance to *B. g. hordei* at the cell periphery (Figure 11B). Interestingly, all tested variants in and adjacent to the SNARE domain resulted in enhanced fungal entry

rates that were each comparable to the partially defective *pen1*-3 mutant, and ranged from 29 (+/-8) % to 60 (+/-8) % (Figure 11B). Three independent lines expressing a PEN1^{I255A} construct showed little variation in fungal entry, ranging from 35 (+/-5) % to 39 (+/-3) % (Figure 11B), whereas a greater level of variation was seen between independent lines expressing PEN1$^{L185A, D186A}$, PEN1$^{I227A, E228A}$ or PEN1$^{A262V, I266A}$. Only few individuals of transgenic lines expressing the PEN1$^{A262V, I266A}$ variant could be analyzed so far. For the PEN1$^{A262V, I266A}$ transformants as well as for PEN1$^{L185A, D186A}$, and for the PEN1$^{I277A, E228A}$, it was difficult to identify individuals expressing wild-type-like levels of the fusion protein. Often protein abundance in the T_2 generation was far below endogenous PEN1 levels and plants were separated out from further analysis. Nevertheless, few transgenic individuals analyzed from three transgenic lines suggest a functional impairment also for the PEN1$^{A262V, I266A}$ variant (Figure 11).

Since the immunoblot analysis is semi-quantitative and eliminated only transgenic lines with PEN1 expression levels that were greater or less than two-fold different from Col-0 wild-type plants, it is possible that subtle variations in the abundance of the respective PEN1 derivatives may result in substantial differences in PEN1 activity.

III.2 Several amino acid substitutions disturb the interaction of PEN1 with the resistance regulator MLO2 *in vivo*

Numerous studies in yeast and animal systems have shown that SNARE protein function is often regulated by additional factors such as SM proteins, Calcium sensor-like synaptotagmin, or Calcium ions (Bock et al. 2001, Gerst 2003, Jahn et al. 2003, Kweon et al. 2003, Marash and Gerst 2003).

In plants, genetic data have revealed a link between the KNOLLE syntaxin and the SM protein family member KEULE because loss-of-function mutations in the respective genes resulted in a phenotypically indistinguishable cytokinesis defect in *Arabidopsis* embryos (Heese et al. 2001). Additionally, the syntaxin mutants barley *ror2* and *Arabisopsis pen1* each partially suppress powdery mildew resistance conditioned by *mlo* mutants in barley and *Arabidopsis,* respectively (Collins et al. 2003, Consonni et al. 2006), which has lead to the hypothesis that wild-type *Hv*Mlo/MLO2 might serve as a negative regulator of *Hv*Ror2/PEN1 syntaxin function.

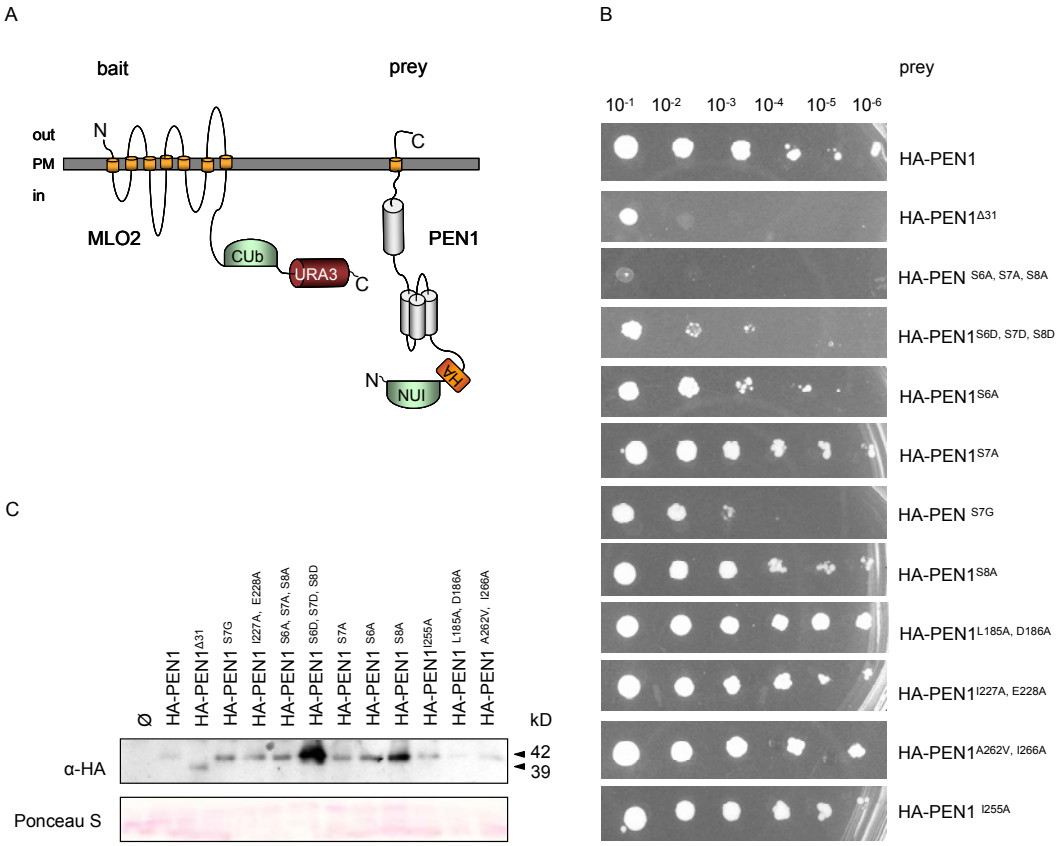


Figure 12. A subset of PEN1 variants fails to interact with MLO2 in yeast. (A) Schematic drawing of MLO bait and PEN1 prey constructs used for yeast transformation. The MLO2 protein was fused to the C-terminal half of ubiquitin (CUb), nd PEN1 wild type and variants were linked to the N-terminus of ubiquitin (NUI). Upon physical interaction of bait and prey, the full-length ubiquitin is reconstituted leading to the degradation of the URA3 (Orotidine-5'-phosphate decarboxylase) reporter enzyme. Consequently the pro-toxin 5-fluororotic acid (FOA) cannot be converted to the toxin, allowing the growth of yeast cells (Stagljar et al. 1998). (B) Yeast cells expressing the MLO2 bait construct were transformed with the PEN1 prey variants and spotted on a selective agar medium (lacking histidine and tryptophane) in the presence of FOA. Yeast colony formation is indicative of physical association between the MLO2 bait and PEN1 or PEN1 variants. The Δ31 non-functional deletion variant of PEN1 was used as a negative control (C.Consonni, unpublished). (C) Immunoblot analysis showing the expression of HA-tagged PEN1 protein variants in yeast. Yeast cells were cultured over night in selective medium and two OD_{600} units of yeast cells were harvested for protein extraction (see Materials and Methods section II.4.1.2). URA3, Orotidine-5'-phosphate decarboxylase; CUb, C-terminal half of ubiquitin; NUI, N-terminal half of ubiquitin.

Because the *Arabidopsis* proteins PEN1 and MLO2 interact in yeast two-hybrid experiments and Förster energy transfer (FRET) between fluorochrome-tagged *Hv*Mlo and *Hv*Ror2 was detected, the genetic link between *Hv*Mlo/MLO2 and *Hv*Ror2/PEN1 has been speculated to reflect a direct physical association between plasma membrane-resident proteins (Panstruga 2005, Schulze-Lefert 2004).

To characterize (*i*) the interaction between PEN1 and MLO2 in more detail, and (*ii*) to examine whether the PEN1 variants that I tested in the transgenic lines (as shown in Figure 10 and Figure 11) enhanced or disrupted the interaction with MLO2, all variants were individually tested for association with MLO2 in the yeast Split-Ubiquitin system (C.Consonni, unpublished; Stagljar et al. 1998). MLO2 fused to the URA3 (Orotidine-5'-phosphate decarboxylase) reporter enzyme was used as a bait construct while wild-type and PEN1 variants were expressed as prey constructs carrying an N-terminal HA-tag for immunodetection to assess prey protein abundance (for details see Figure 12A). Yeast cells transformed with the MLO2-URA3 bait constructs are able to grow on uracil-lacking selective medium, which was used to observe the expression of the bait construct. I monitored growth of co-transformed yeast cells on selective 5-fluoroorotic-acid (FOA)-containing medium. Wild-type PEN1 prey co-transformed with the MLO2 bait supports yeast growth on FOA-containing selective medium even at high dilutions of the yeast cell inoculum (10^{-6}; Figure 12B). As a negative control PEN1$^{\Delta 31}$ was used, which is a non-functional variant of PEN1, carrying a 31 amino acid in-frame deletion known to result in the loss of interaction with MLO2 in yeast (C. Consonni, unpublished). As expected, yeast growth was absent in cells co-expressing the MLO2 bait and the PEN1$^{\Delta 31}$ construct (Figure 12B).

Interestingly, several PEN1 variants carrying substitutions at the N-terminal serines residues (S6, S7, and S8) did not support full growth of yeast cells. For PEN1$^{S6A,\ S7A,\ S8A}$ yeast growth was almost completely absent, comparable to PEN1$^{\Delta 31}$, while for PEN1$^{S6D,\ S7D,\ S8D}$ and PEN1^{S7G} a clear reduction of yeast growth was observed. Single exchanges of S6, S7 or S8 to alanine did not have an effect on yeast growth. In addition, neither of the PEN1 prey variants carrying substitutions in the SNARE domain or in adjacent sequences resulted in diminished yeast growth. To assess whether lack of yeast growth resulted from instability or absence of the PEN1 variants, immunoblot analysis was performed (Figure 12C). All PEN1 prey proteins were detectable in yeast extracts although the abundance of individual PEN1 differed greatly in a few cases (e.g. wild-type PEN1 compared to PEN1$^{S6D,\ S7D,\ S8D}$; see Figure 12C). The lowest protein

abundance was detected for the prey construct carrying wild-type PEN1 and PEN1$^{L185A, D186A}$ sequence, the highest for PEN1$^{S6D, S7D, S8D}$. Since wild-type PEN1 and PEN1$^{L185A, D186A}$ were both sufficient to support yeast growth on selective medium even at a dilution of 10^{-6} of the yeast cells and the latter, PEN1$^{S6D, S7D, S8D}$, only up to a dilution of 10^{-3}, a direct correlation between prey abundance and yeast growth in the presence of FOA can be excluded. Consequently, N-terminal serine residues of PEN1 seem to play an important role for interaction with MLO2 in this heterologous yeast-based assay.

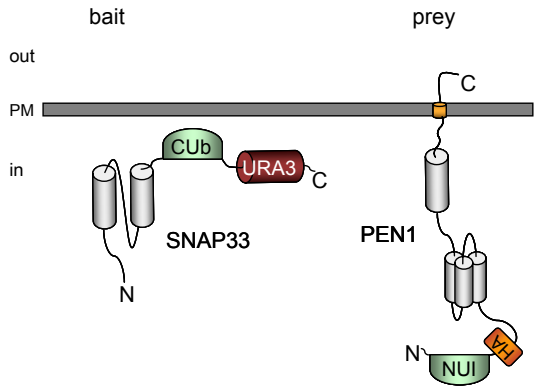


Figure 13. PEN1 and PEN1 variants fail to interact with SNAP33 in the yeast split ubiquitin system. Schematic drawing depicting the domain structures of SNAP33-Cub-URA bait construct and the PEN1-HA-NUI prey constructs. Note that the Cub fused to the C-terminus of SNAP33 might be spatially separated from NUI at the N-terminus of the PEN1 prey construct upon N-to N-orientation of the SNARE domains during binary complex formation preventing reconstitution of a functional ubiquitin (detail see text).

To test whether specific amino acid residues are required for PEN1 interaction with the *Arabidopsis* SNAP25-like protein SNAP33, a SNAP33 bait construct was generated (Figure 13). Although the SNAP33 bait construct was expressed in yeast cells indicated by the growth of the transformed yeast cells in the absence of uracil, co-expression with all tested PEN1 prey constructs did not support yeast growth on selective FOA containing medium (data not shown). This was rather unexpected since previous yeast two-hybrid studies using SNARE proteins lacking the transmembrane-helix have revealed evidence for protein-protein interactions of SNAREs (e.g. Collins et al. 2003, Hata and Südhof 1995, Widberg et al. 2003). Indeed, the so-called binary complex

formation between a SNAP25-like protein and a syntaxin is thought to follow a zipper mechanism starting from electrostatic interactions between N-terminal SNARE domain residues (Fasshauer and Margittai 2004, Pobbati et al. 2006). This requires the N-to N-orientation of SNARE domains. Since the Cub-URA3 reporter construct is fused to the C-terminus of SNAP33 it might become sterically oriented relative to the NUb in a way that does not permit reconstitution of Ubiquitin (see Figure 13). Thus the lack of yeast growth may be due to sterical hindrance of bait and prey constructs.

III.2.1 Site-directed PEN1 variants do not restrict fungal ingress of the adapted *Arabidopsis* powdery mildew pathogen *E. cichoracearum*

A genetic link between *MLO2* and *PEN1* has been shown in the interaction of *Arabidopsis* with the pathogenic powdery mildew *E. cichoracearum* (Consonni et al. 2006). To test if the site-directed PEN1 variants displaying an altered interaction with MLO2 in the yeast two-hybrid assay, would also produce an altered infection phenotype with the host fungus, a pilot experiment was conducted with the support of our collaborators at Standford University (M.Lim, B.Hou and S.Somerville).

The tested transgenic lines showed no recognizable differences of *E. cichoracearum* entry rates compared to Col-0 wild type (Figure 14). *Arabidopsis mlo2* single mutants were significantly more resistant than Col-0 wild-type plants to both tested powdery mildews, restricting *E. cichoracearum* ingress to 35 (+/-10) % compared to 87 (+/-6) % in wild type plants and fungal entry of *B. g. hordei* conidiospores to 10 (+/-1) % compared to 15 (+/-1) % in Col-0 wild type (see Figure 14), which is consistent with previously published findings (Consonni et al. 2006). The double mutant *mlo2*-11/*pen1*-1 allows 47 (+/-8) % of fungal entry of *B. g. hordei* spores (Figure 14). These data support previous findings of an antagonistic genetic link between *MLO2* and *PEN1* in interactions with the adapted powdery mildew *E. cichoracearum* (Collins et al. 2003, Consonni et al. 2006) and reveal an additional role of this link in the interaction with the non-host fungus *B. g. hordei.*

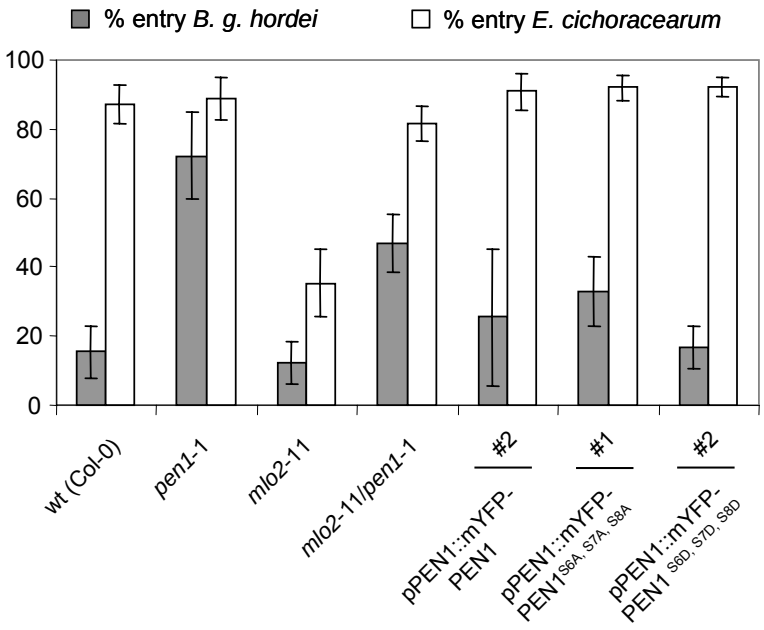


Figure 14. Differential restriction of fungal entry in host and non-host powdery mildew interactions. Fungal entry into leaf epidermal cells was measured at 72 hpi for *B. g. hordei* (grey bars) and at 48 hpi for *E. cichoracearum* (white bars). White bars represent data from a single pilot experiment using six individual plants per genotype. Grey bars represent at least 22 individuals from at least six independent biological replicates; the data was re-plotted from Figure 10. Error bars represent either SEM for *B. g. hordei* inoculations, or standard deviations for the *E. cichoracearum* experiment. n, number of individual plants tested.

III.3 Functional diversification of PEN1 and SYP122

SYP122 is the closest relative of *PEN1* among the *Arabidopsis* syntaxins. The two proteins share an overall sequence identity of 63%; in the SNARE domain sequence identity is 76%. The *syp122*/*pen1* double mutant is severely dwarfed and shows leaf necrosis (Assaad et al. 2004, Zhang et al. 2007). Both necrosis and dwarfism of the double mutant are to a large part mediated by de-regulated high levels of the defense signaling molecule salicylic acid (SA), because *pen1/syp122* plants carrying an additional mutation in *NPR1* or *EDS1,* genes required for SA-dependent pathogenesis-related (*PR*) gene expression and signaling, respectively (Cao et al. 1997, Wang et al. 2006, Wiermer et al. 2005), *SID2*, a gene required for the biosynthesis of SA

(Wildermuth et al. 2001), or carrying the bacterial *NahG* transgene encoding a salicylate hydrolase, which prevents accumulation of SA by immediate conversion to catechol (You et al. 1991), grow similar to wild-type plants (Zhang et al. 2007). Absence of detectable necrosis and dwarfism in *pen1* and *syp122* single mutants suggests that the two proteins may have additional potentially overlapping functions (Assaad et al. 2004, Zhang et al. 2007). Since screening for mutants altered in non-host resistance responses to *B. g. hordei* in an EMS-mutagenized *Arabidopsis* population recovered four mutant alleles of the *PEN1* syntaxin (*pen1*-1 to *pen1*-4; Collins et al. 2003, Zhang et al. 2007) and several other *PENETRATION* genes (PEN1 to PEN4; see Introduction section I.4.1; Lipka et al. 2005, Stein et al. 2006; M. Lim and S. Somerville unpublished) but no other *Arabidopsis* syntaxins, it is likely that *PEN1* and *SYP122* are functional diversified with regard to a disease resistance activity against non-adapted powdery mildews at the cell periphery (Assaad et al. 2004, Collins et al. 2003).

III.3.1 PEN1 and SYP122 proteins are upregulated during pathogenesis

To elucidate whether differences in protein abundance of the two-related *Arabidopsis* PEN1 and SYP122 syntaxins during fungal infection could account for their functional specialization, a time course experiment was performed (Figure 15). PEN1 protein was detected in unchallenged leaf tissue and at early time points after *B. g. hordei* spore inoculation using PEN1-antiserum (12 hpi). In contrast, steady state levels of SYP122 in unchallenged tissue were barely detectable using a SYP122-antiserum (provided by T. Nühse). While PEN1 protein abundance increased only slightly between 12 and 24 hpi, SYP122 showed a strong transient increase in protein abundance at 24 hpi and declined thereafter (Figure 15B). This correlates well with previous reports demonstrating that S*YP122* transcriptional upregulation during pathogen challenge is more pronounced than *PEN1* responsiveness (Assaad et al. 2004). Note that PEN1 protein levels remained unchanged at 24 hpi (Figure 15A). Although PEN1 levels appear wild-type-like in *syp122*-1 mutants at any time point sampled (Figure 16A), the levels of SYP122 appear to increase at 51 hpi compared to wild-type plants in *pen1*-1, *pen1*-3, and *mlo2*-6/*pen1*-1 double mutants (Figure 16B). Thus, *Arabidopsis* plants appear to compensate for the loss of PEN1 by increasing SYP122 abundance during pathogen challenge, but not vice-versa.

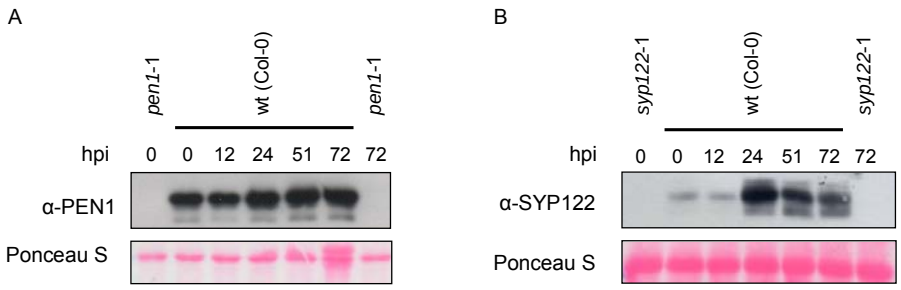


Figure 15. PEN1 and SYP122 protein levels increase after challenge with non-adapted *B. g. hordei* spores. Time course experiments were preformed and protein abundance of PEN1 and SYP122 were tested by immunoblot of total leaf extracts using PEN1- and SYP122-antisera following SDS-polyacrylamide gel electrophoresis. Leaf samples of wild type (Col-0) and the respective null mutant control plants (*pen1*-1 and *syp122*-1) were taken before (0 hpi) and after 12, 24, 51 and 72 hpi spore inoculation with *B. g. hordei*. (A) Immunoblot of total leaf extract probed with PEN1-antiserum. Per lane 5 µg of protein of total leaf extract was loaded. PEN1 protein levels increase slightly between 12 and 24 hpi and remain elevated also late during infection at 72 hpi. (B) Immunoblot of total leaf extract probed with SYP122-antiserum (Nuhse et al. 2003). Per lane 30 µg of protein of total leaf extract was loaded. SYP122 protein levels increase at least ten-fold at 12 to 24 hpi, followed by a decline in abundance at 51 and 72 hpi.

The compensatory effect was particularly striking in *mlo2*-6 single and *mlo2*-6/*pen1*-1 double mutants in which SYP122 was detected at 12 hpi and even in the absence of the pathogen, respectively (Figure 16 B). It seems unlikely that the observed pathogen induced compensatory upregulation of SYP122 at late time points after spore inoculation (> 24 hpi), influences *B. g. hordei* entry into leaf epidermal cells since this step of *B. g. hordei* pathogenesis takes place around 10-15 hpi (reviewed in Thordal-Christensen et al. 2000). However, in the interaction with adapted powdery mildews, *pen1*-1 plants support wild-type-like entry rates (80%; see Figure 14; Lipka et al. 2005). Whether elevated levels of SYP122 in *pen1*-1 mutants might be responsible for restricting pathogenesis of adapted powdery mildews to wild-type-like levels by restricting secondary entry attempts of adapted powdery mildews remains to be shown.

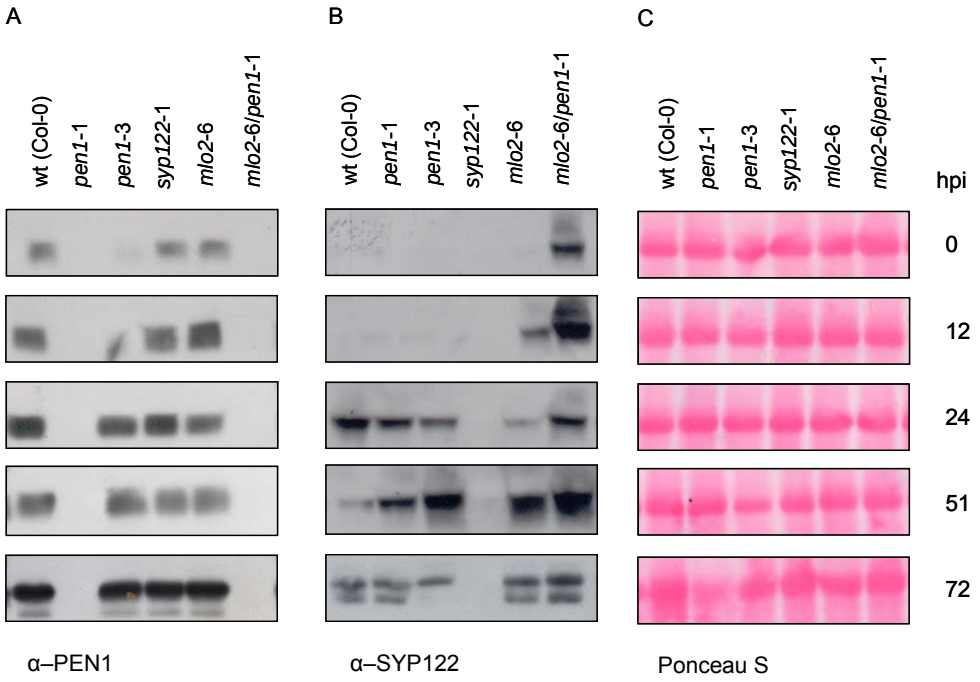


Figure 16. SYP122 protein levels in *mlo2* and *pen1* mutants are highly pathogen responsive. Time course experiment assessing PEN1 and SYP122 protein levels in total leaf extract during infection. (A) Detection with PEN1-antiserum. (B) Detection with SYP122-antiserum (Nuhse et al. 2003). (C) Ponceau S staining to demonstrate equal loading.

III.3.2 Does differential protein abundance of PEN1 and SYP122 syntaxins account for PEN1-specific activity in disease resistance at the cell periphery?

To analyze whether elevated steady state SYP122 levels could complement for the impaired pre-invasion non-host resistance in *pen1*-1 plants, I generated transgenic plants carrying a p35S::cCFP-SYP122 construct in a *pen1*-1 background. In addition, I transformed a "promoter swap" construct driving *SYP122* cDNA with the PEN1 promoter sequence (pPEN1::mYFP-SYP122) in *pen1*-1 null mutant plants. These two constructs should reveal whether the functional diversification between PEN1 and SYP122 in disease resistance depends on differences at the level of gene regulation and/or leading to differences in protein abundance at the time of fungal attack.

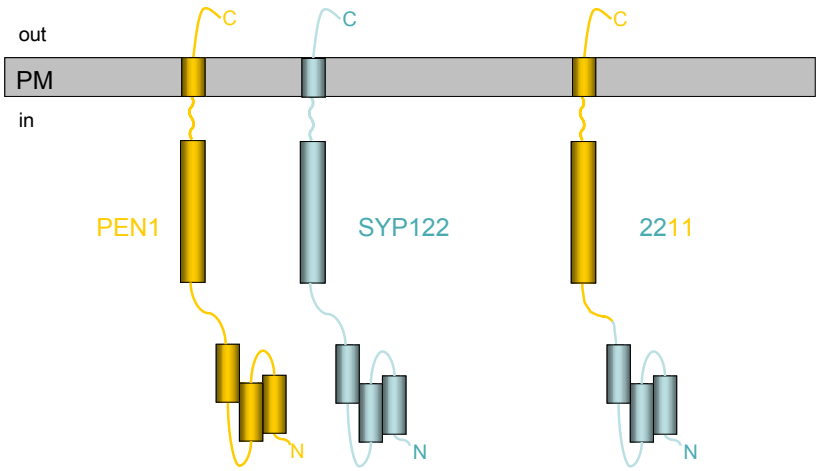


Figure 17. A syntaxin chimera generated by swapping the N-terminus of PEN1 with SYP122. Schematic drawing depicting the domain structures of PEN1 (in yellow) and SYP122 (in blue) syntaxins. The syntaxin chimera comprises the first 175 amino acids of SYP122 and the N-terminus (amino acids 176 - 346) of PEN1 as indicated in yellow and blue color.

To examine whether functional specialization is determined by differences in the amino acid sequences of PEN1 and SYP122, a syntaxin chimera was generated that consisted of the regulatory N-terminal part of SYP122 (methionine 1 to valine 175) and C-terminal PEN1 sequence (threonine 176 to arginine 346; Figure 17). The corresponding constructs were tested for the ability to rescue the *pen1*-1 mutant phenotype by *B. g. hordei* spore inoculation experiments (Figure 19).

III.3.3 High steady stat levels of SYP122 fail to complement for the loss of PEN1

Surprisingly, expression of SYP122 derivatives in *pen1*-1 plants lead to a dwarfed and necrotic phenotype in several cases, which was reminiscent of the phenotype described for *pen1*-1/*syp122*-1 double mutant plants (Assaad et al. 2004; see Figure 18). Six plants carrying the pPEN1::mYFP-SYP122 construct were identified as positive for mYFP-fluorescence in the T_1 generation. Two of these showed a dwarf phenotype at later stages of development (> 3 weeks).

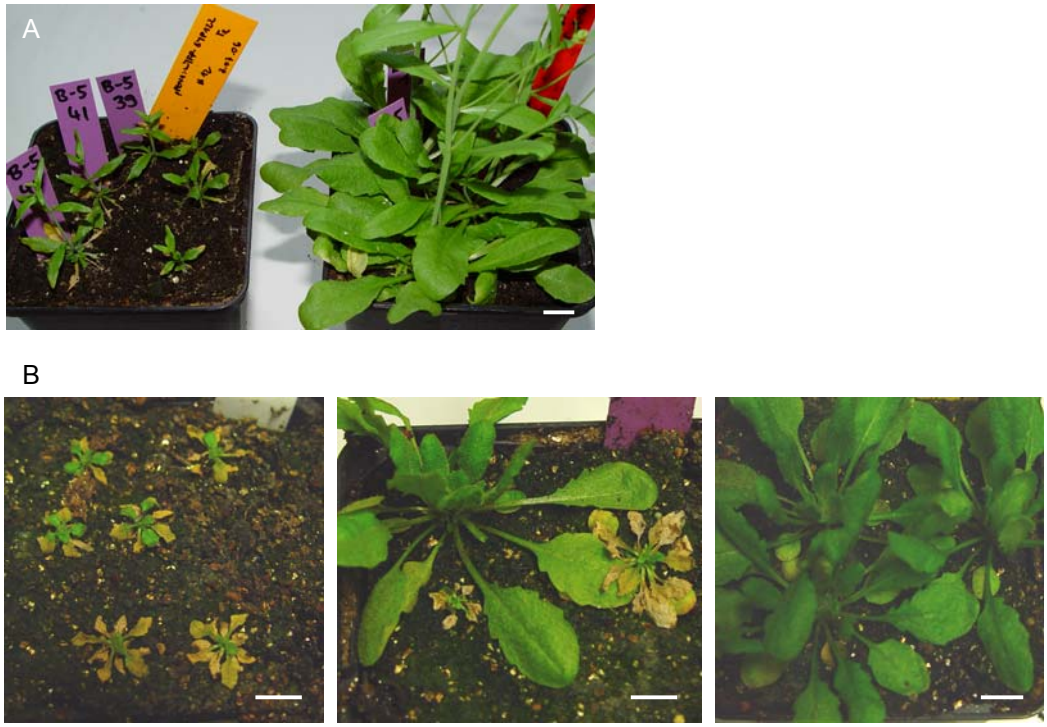


Figure 18. Expression of *SYP122* in a *pen1*-1 mutant background may lead to dwarfism and leaf necrosis. (A) Six week-old *pen1*-1 plants transformed with a pPEN1::mYFP-SYP122 construct (left) are shown in comparison to wild-type plants (right). (B) Four week-old plants expressing SYP122 from the 35S promoter in *pen1*-1 background are shown. T_2 plants carrying the overexpression p35s::cCFP-SYP122 construct. All plants show the dwarfism and leaf necrosis (left photograph). Immunodetection of SYP122 fusion protein failed (not shown). Transgenic line #55 containing p35S::CFP-SYP122 in the *pen1*-1 background provided by F. Assaad (middle photograph). A dwarfed and necrotic phenotype was observed occasionally (one in nine plants). Plants with wild-type-like growth expressed the fusion protein to detectable levels (see Figure 19).

For the chimeric constructs, dwarf phenotypes were observed irregularly (10 to 90% depending on the individual transgenic lines) in the T_2 generation. To date, I failed to identify plant lines stably expressing SYP122 or syntaxin chimera driven by the PEN1 5' regulatory sequences. Similarly, I failed to detect lines stably expressing p35S::cCFP-SYP122 among recovered T_1 plants. Fortunately, an alternative p35S::CFP-SYP122 overexpression line in a *pen1*-1 background was provided by F. Assaad (Assaad et al. 2004).

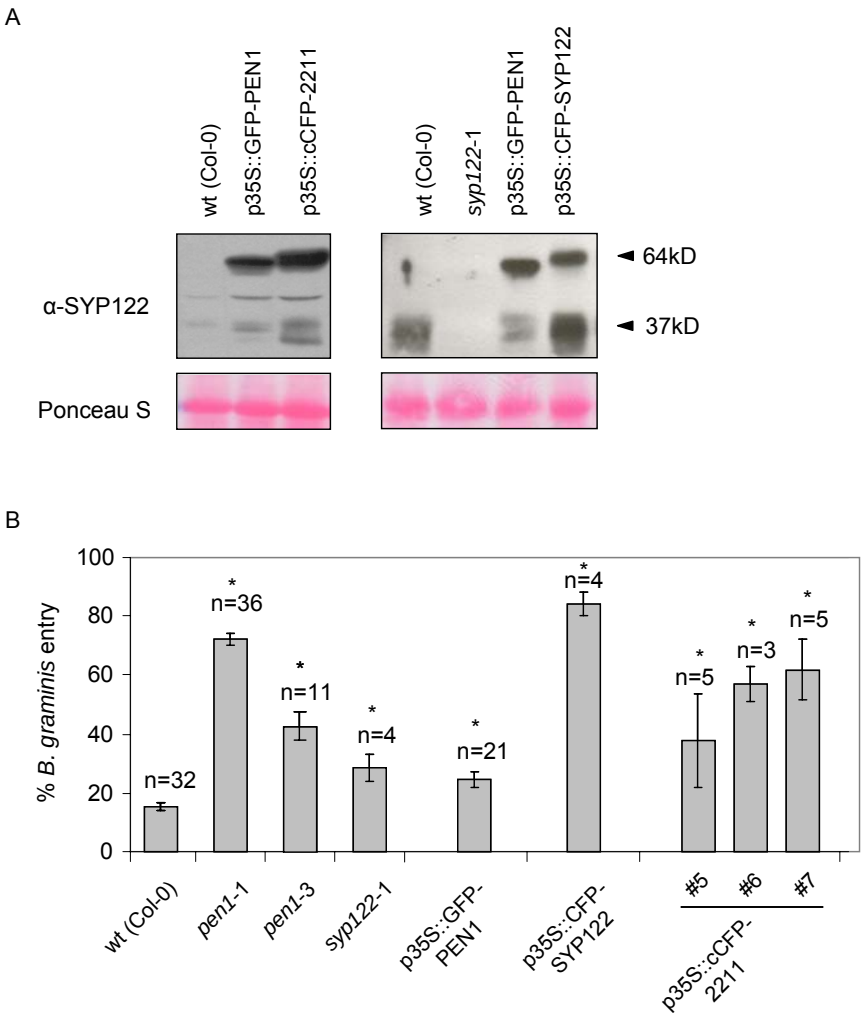


Figure 19. Overexpression of SYP122 or a SYP122-PEN1 chimera does not rescue the *pen1*-1 phenotype. (A) Immunoblot analysis of three- to five-week old plants 72 hpi using SYP122-antiserum (Nuhse et al. 2003). (B) The overexpression line p35S::CFP-SYP122 in *pen1*-1 (Assaad et al. 2004) and three independent lines (#5, #6, #7) expressing the domain syntaxin chimera 2211 in *pen1*-1 were analyzed for complementation of the *pen1* entry phenotype to *B. g. hordei*. Note: p35S::CFP-SYP122 plants were five weeks-old by the time of analysis, which most likely results in the unusually high entry rates. Consistent with this *pen1*-1 control plants sampled at identical age showed comparably high entry rates (84%). n, number of individual plants tested.

Siblings of this line occasionally showed a dwarf phenotype (in about one out of nine plants; Figure 18). Several lines were identified expressing the syntaxin chimera 2211. Also these lines showed occasional dwarfism and necrosis (approximately one in five

plants in all tested lines). Plants exhibiting wild-type-like growth of each, SYP122 and syntaxin chimera expressing lines, were analyzed for the functionality of the transgenes in spore inoculation experiments with *B. g. hordei* (Figure 19).

Complementation of the enhanced *pen1*-1 fungal entry phenotype in plants expressing the p35S::CFP-SYP122 or the syntaxin chimera in a *pen1*-1 background was assessed as described before. Although the fusion proteins were detectable on immunoblots of SDS-polyacrylamid separated total leaf extract and exceeded SYP122 wild-type levels approximately ten-fold (Figure 19A), none of the tested constructs were able to rescue the enhanced fungal entry phenotype (Figure 19B). These findings indicate that PEN1 and not SYP122 contribute to pre-invasion penetration resistance to non-adapted powdery mildews. Concerning non-host immunity to powdery mildews, functional diversification between the two related syntaxins can be assumed to be complete.

III.4 Subcellular localization of PEN1, SNAP33, and VAMP722 SNARE proteins in plant defense responses

III.4.1 Fluorochrome-tagged PEN1 is functional and accumulates underneath attempted *B. g. hordei* entry sites

Fluorochrome-tagged PEN1 has been used previously to study protein localization in pathogen-challenged and unchallenged leaf tissue at subcellular resolution (Assaad et al. 2004, Bhat et al. 2005, Collins et al. 2003). These authors used the strong Cauliflower mosaic virus 35S promoter (Sanders et al. 1987) to drive fusion protein expression. It was shown that p35S driven GFP-PEN1 fusion protein localizes to the plasma membrane and focally accumulates underneath powdery mildew appressoria (Assaad et al. 2004, Bhat et al. 2005, Collins et al. 2003). Additionally, several other plasma membrane anchored marker proteins tagged by a fluorochrome show a similar behaviour (Koh et al. 2005, Stein et al. 2006). Their contribution to disease resistance is not known, except for PEN3, an ATP-binding cassette transporter, which has been identified to contribute to non-host resistance to non-adapted pathogens (Stein et al. 2006). Except for PEN3-GFP, and the receptor kinase BRI1-GFP, required for brassinosteroid sensing in *Arabidopsis* (Li and Chory 1997), which were both driven by native regulatory sequences, the fluorochrome-tagged marker proteins that were

reported to accumulate beneath attempted fungal entry sites were expressed from the strong 35S promoter, and it cannot be excluded that their apparent focal accumulation may be an ectopic localization resulting from overexpression. To re-examine this for PEN1, 1,2 kb of the native upstream regulatory sequence of PEN1 (pPEN1; designated "native promoter") was used to drive the expression of a monomeric yellow fluorescent protein (mYFP; Zhang et al. 2002) fused to the N-terminus of PEN1. The depicted domain structure of the fusion protein is illustrated in Figure 20A. In addition to this pPEN1::mYFP-PEN1 construct, *Arabidopsis* plants expressing a cCFP- and a mYFP-PEN1 fusion construct under the control of the 35S Cauliflower mosaic virus promoter were generated to allow for co-localization studies (see Table 21 and 0).

PEN1 subcellular localization at endogenous expression levels is shown in Figure 20. The fusion protein localizes to the cell margins and the fluorescent signal retracts with the plasma membrane during plasmolysis experiments, thereby pointing to an association with the plasma membrane (Figure 20B and C). Analysis of transgenic lines overexpressing fluorochrome-tagged PEN1 under the control of the 35S promoter revealed Hechtian strands during plasmolysis as shown in Figure 20C (and Collins et al. 2003). The formation of Hechtian strands strongly supports PEN1 plasma membrane localization (Hecht 1912, Oparka 1994). Such thin plasma membrane strands were rarely detectable in pPEN1::mYFP-PEN1 expressing lines (data not shown). This might be due to an insufficient mYFP-PEN1 fluorochrome abundance in these lines to visualize fluorescent signals originating from Hechtian strands. However, *B. g. hordei*-induced focal accumulation of mYFP-PEN1 underneath attempted entry sites was clearly detectable in pPEN1::mYFP-PEN1 native promoter lines (Figure 20D).

Taken together, there were no recognizable differences in subcellular localization or re-localization of PEN1 upon powdery mildew challenge between native promoter lines and 35S overexpression lines, confirming previously published data (Assaad et al. 2004, Bhat et al. 2005, Collins et al. 2003). My data extend previous studies with PEN1 fusion proteins and show that N-terminally fluorochrome-tagged PEN1 fusion proteins are functional in disease resistance, when expressed at wild-type levels (Figure 9).

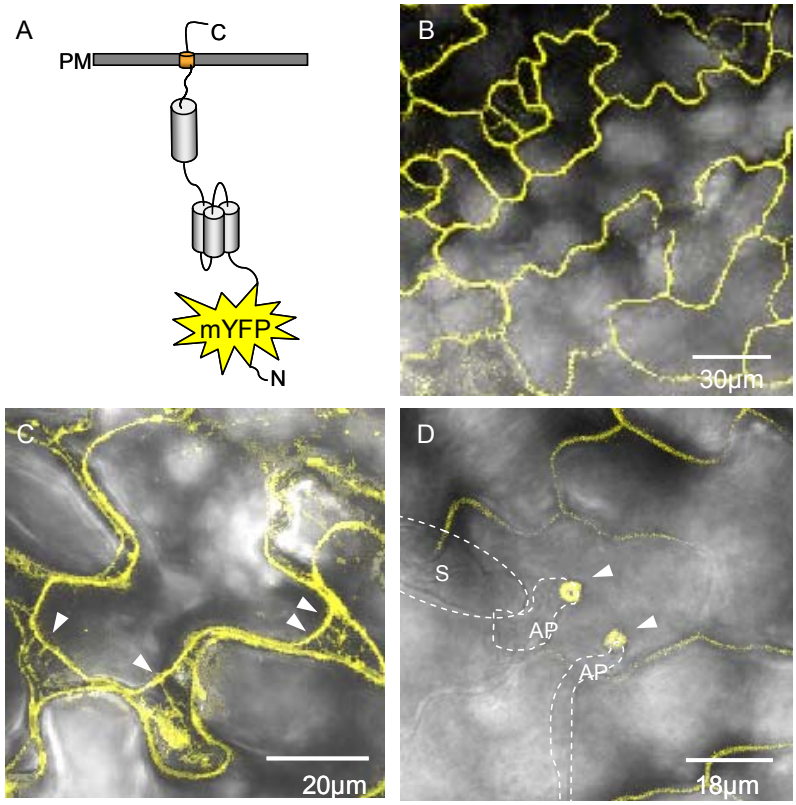


Figure 20. pPEN1 driven mYFP-PEN1 localizes to the plasma membrane and focally accumulates underneath fungal appressoria. Leaves of ten days old *Arabidopsis* plants expressing pPEN1::mYFP-PEN1 or p35S::mYFP-PEN1 in a *pen1* null mutant background were inspected by confocal microscopy. (A) Schematic drawing of the expected domain structure of mYFP-PEN1 anchored to the plasma-membrane. (B) pPEN1::mYFP-PEN1 expressing plants show a mYFP-PEN1 fluorescence signal in the plasma membrane of leaf epidermal cells. Protein levels were similar to wild type as examined by immunoblotting depicted in Figure 8B. Excitation of mYFP was achieved at 514 nm and fluorescence emission was detected at 518 to 578 nm (C) p35S::mYFP-PEN1 expressing plants were imaged during plasmolysis (5M sorbitol, 30 min) to visualize Hechtain strands. The mYFP-PEN1 signal retracts from the cell wall. Hechtian strands are formed and indicated by arrow heads. Hechtian strand formation was rarely seen with pPEN1::mYFP-PEN1 lines, details see text. (D) pPEN1::mYFP-PEN1 plants 18 h after spore inoculation with *B. g. hordei*. Ring-shaped focal accumulations of mYFP-PEN1 (indicated by arrow heads) are visible underneath the two fungal appressoria (AP). Spore (S) and appressoria are outline by dashed white lines.

In addition, the subcellular localization of the PEN1 variants analyzed in this study in healthy and powdery mildew challenged leaf cells was indistinguishable from the corresponding wild-type PEN1 construct, as well as the mYFP-PEN1-3 fusion protein. Thus, none of the amino acid exchanges introduced into PEN1 altered its ability to accumulate underneath fungal entry sites (data not shown).

III.4.2 PEN1 SNARE partners are recruited to sites of fungal attack

SNARE proteins are known to play key roles in vesicle trafficking and formation of cognate ternary SNARE complexes has been, at least partially, accounted for the specificity of the fusion of vesicles at target membranes (reviewed in Bonifacino and Glick 2004, Hong 2005). Typically, exocytotic ternary SNARE complexes consist of one member of the SNAP25 family, one member of the syntaxin family and one v-SNARE, i.e. VAMP protein family member (see Introduction I.1.; reviewed in Li and Chin 2003). If *Arabidopsis* SNAP25 and VAMP family members form a ternary complex with PEN1 in disease resistance, then one would expect these proteins to localize to the same subcellular compartment.

The Arabidopsis genome comprises three *SNAP25*-like SNARE family members, *SNAP29, SNAP30* and *SNAP33* (Sanderfoot 2007, Sanderfoot et al. 2000, Uemura et al. 2004). Gene expression studies indicate that *SNAP33* is the only *SNAP25*-family member of *Arabidopsis* expressed in significant amounts in leaf tissue (C. Neu, unpublished; Wick et al. 2003). Furthermore, SNAP33 transcript and protein levels are known to be upregulated upon inoculation with the various plant pathogens, i.e. the fungus *Plectosporium tabacum,* the oomycete *Peronospora parastica* and the bacterial pathogen *Pseudomonas syringae* (Wick et al. 2003). We therefore considered SNAP33 as candidate interaction partner of the PEN1 syntaxin.
Arabidopsis possesses 14 *VAMP* encoding genes (Sanderfoot et al. 2000, Uemura et al. 2004). This exceeds the numbers identified in the genomes of other higher eukaryotes, such as the human genome in which nine VAMPs have been identified (Sanderfoot 2007). Amongst the *Arabidopsis* VAMPs, VAMP722 and the highly sequence related VAMP721 are the only VAMPs shown to localize to the plasma-membrane in a transient expression system utilizing protoplasts of cultured *Arabidopsis* cells (Uemura et al. 2004).

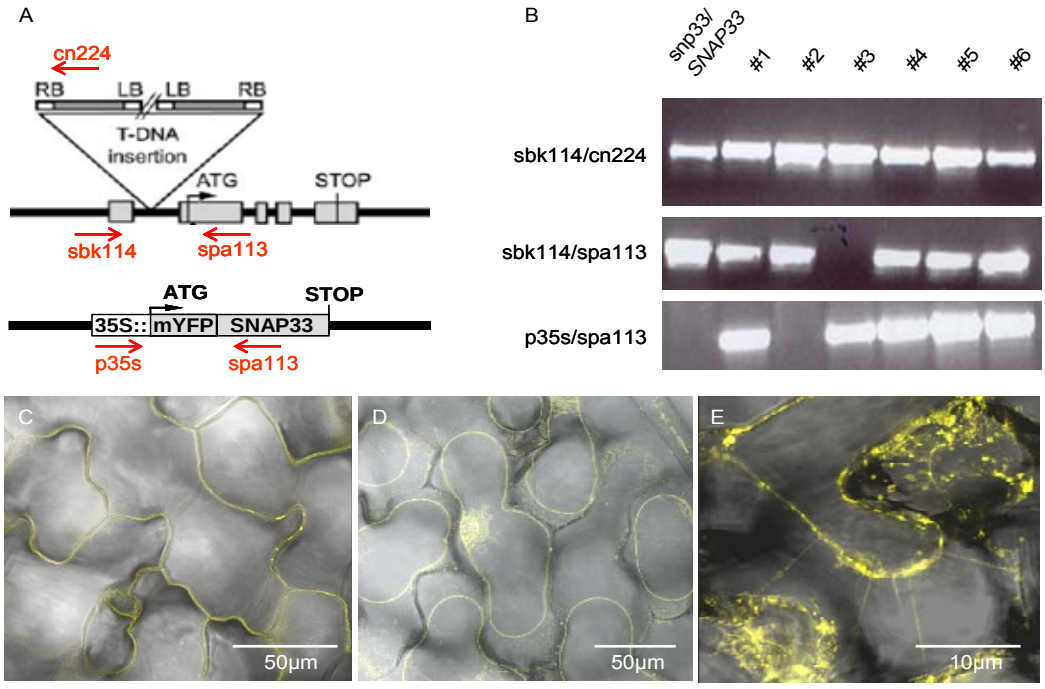


Figure 21. A mYFP-SNAP33 fusion construct complements *snp33* mediated embryo lethality and associates with the plasma membrane. Heterozygous *snap33/SNAP33* mutant plants were transformed with a mYFP-SNAP33 fusion construct driven by the 35S promoter. (A) Schematic drawing of the SNAP33 T-DNA insertion (modified from Heese et al. 2001) and the transgene construct. (B) Six independent T_1 plants (#1- #6) were selected and genotyped by PCR. To identify transformed lines that are homozygous for the T-DNA insertion the indicated primer combinations sbk114/cn224 (upper panel) and sbk114/spa113 (central panel) were used. An additional PCR (lower panel) was preformed using transgene specific primers (p35s/spa113) to test for successful transformation. The relative primer annealing positions are depicted in (A). (C) Confocal images of leaf epidermal cells of complementation line #3. Images were taken at 514 nm to excite mYFP and emission was measured at 518 to 578 nm. mYFP-SNAP33 fluorescence indicates localization of the fusion protein to the plasma membrane. The fluorescent signal retracts with the plasma membrane during plasmolysis and can be seen in Hechtian strands (H, right picture).

Transient *VAMP722* gene expression by biolistic gene delivery into barley leaf epidermal cells has revealed that VAMP722 accumulates at attempted *B. g. hordei* entry sites in this heterologous experimental system (S.Bau, unpublished). This could be confirmed in stable transgenic lines expressing mYFP-VAMP722 in a *vamp722* null mutant background under the control of the 35S promoter (C. Neu, unpublished).

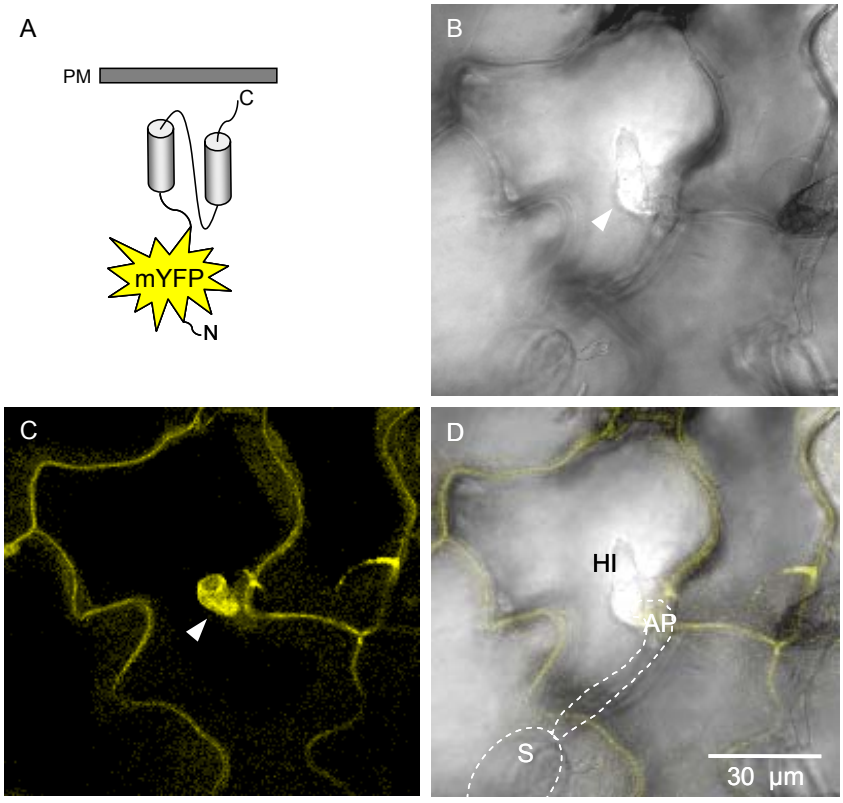


Figure 22. mYFP-SNAP33 accumulates beneath *B.graminis* appressoria. *Arabidopsis* plants expressing mYFP-SNAP33 in a *snp33* null mutant background (line #3, see Figure 21) were analyzed microscopically after *B. g. hordei* spore inoculation. (A) Schematic drawing depicting the domain structure of mYFP-tagged SNAP33. (B) A fungal spore successfully entered a leaf epidermal cell and accommodated a haustorial initial (as indicated by the arrow head). The light microscopic confocal image was taken using the transmission channel. (C) A typical cup-shaped PEN1 accumulation structure previously described at haustorial initials (as indicated by the arrow head). Images were taken at 514 nm to excite mYFP and emission was detected at 518 to 578 nm. (D) Overlay of (B) and (C). HI-haustorial initial, AP appressoria, S spore.

To date, neither plant ternary SNARE complexes have been reported, nor do genetic data suggest cognate combinations of SNARE genes that might point to ternary SNARE complexes in a physiological process (Heese et al. 2001, Schulze-Lefert 2004). Recently, a pathogen-induced SDS-resistant and heat-labile PEN1 containing SNARE complex was isolated from plant leaf tissue in our lab (Kwon et al. in preparation). This complex was also shown to contain SNAP33. *In vitro*, PEN1, SNAP33 and members of

the VAMP72 group engage in ternary SNARE complexes (Kwon et al. in preparation). Interestingly, *pen1*-3, a partial defect allele of PEN1 initially observed by Collins et al., which carries a glycine to aspartate exchange in the conserved SNARE domain of the protein (G269E; see alignment in Figure 7, chapter III), displayed a selective defect in ternary complex formation with VAMP722, but not with other members of the VAMP72 group tested. Furthermore, simultaneous transcript-based depletion of both, *VAMP721* and *VAMP722* in transgenic *Arabidopsis* plants revealed enhanced *B. g. hordei* entry in leaf epidermal cells similar to the *pen1*-1 phenotype (Kwon et al. in preparation).

To substantiate these findings, I tested for powdery mildew-induced accumulation of these candidate SNARE partners of PEN1. Transgenic *Arabidopsis* plants expressing mYFP-tagged SNAP33 and, in cooperation with C. Neu, mYFP-VAMP722 were inspected. For this purpose, I transformed a 35S promoter construct driving mYFP-tagged SNAP33 in *Arabidopsis* plants that are heterozygous for a T-DNA insertion in *SNAP33* (*snp33/SNP33*; Figure 21).

Homozygous *snp33* knock-out plants are known to be embryo-lethal (Heese et al. 2001). T_1 plants were screened for expression of the fluorochrome-tagged protein by fluorescence microscopy. Six plants were identified showing mYFP-fluorescence in leaf epidermal cells and genotyped by PCR using primer combinations that report the presence or the absence of the T-DNA insertion and the transgene (Figure 21A and B). Among these, one plant line (#3) was found to be homozygous for the T-DNA insertion. This indicates that the fusion protein expressed from the p35S::mYFP-SNAP33 construct is able to rescue the embryo-lethality of homozygous *snap33* mutants. It is therefore reasonable to assume that the fusion protein is also functional in disease resistance responses to powdery mildew parasites. Upon fungal challenge, mYFP-SNAP33 accumulates at sites of attempted *B.graminis* attack, i.e. at fungal haustorium initials, which is reminiscent of the PEN1 focal accumulation described above (Figure 22).

III.4.3 Powdery mildew induced co-localization of *Arabidopsis* SNARE partner proteins

To directly explore whether the timing of powdery mildew-induced accumulation of PEN1 and its putative SNARE partner proteins is similar and occurs at the same

position underneath fungal appressoria, I generated plants expressing cCFP-tagged PEN1 and crossed these with lines expressing mYFP-tagged SNAP33 or VAMP722. The resulting two colour lines, co-overexpress either cCFP-PEN1 and mYFP-SNAP33; or cCFP-PEN1 and mYFP-VAMP722. A summary of the examined transgenic lines, subcellular localization of the fusion proteins in healthy leaf epidermal cells and accumulation beneath powdery mildew appressoria is shown in Table 21.

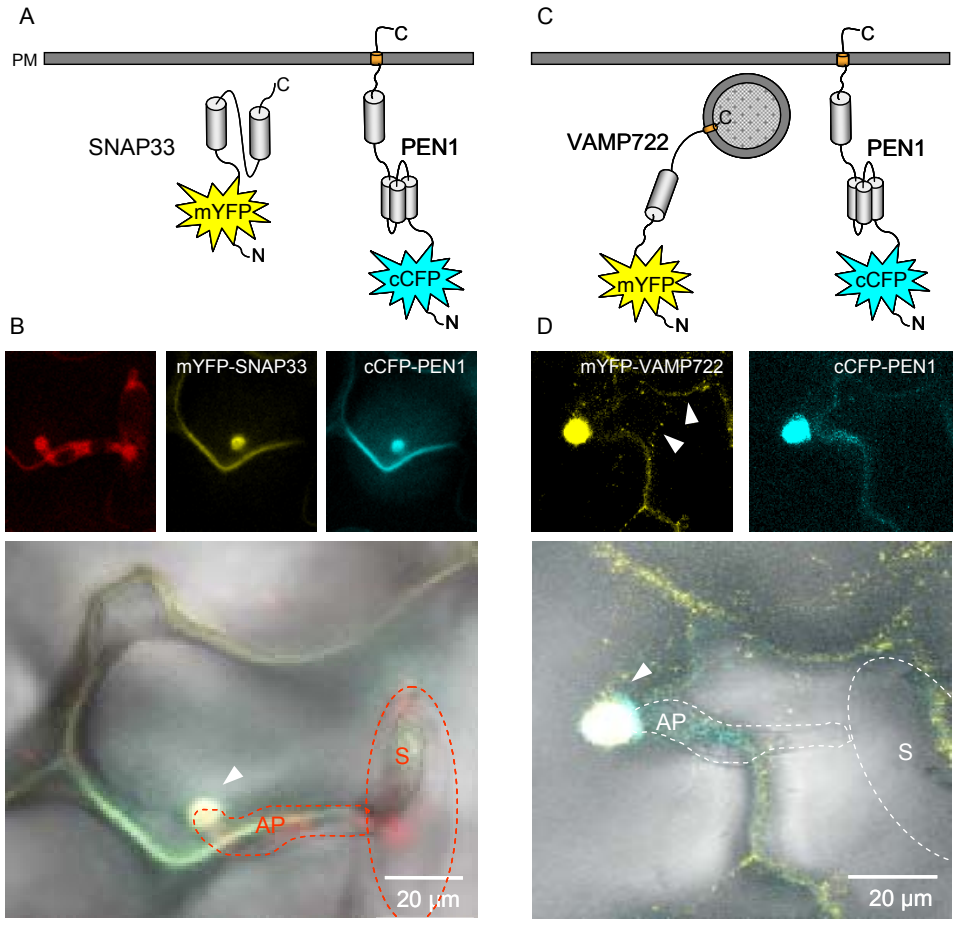


Figure 23. SNAP33 and VAMP722, potential SNARE interaction partners of PEN1, focally accumulate underneath fungal appressoria and co-localize with PEN1. *Arabidopsis* lines co-expressing either cCFP-PEN1 and mYFP-SNAP33 or cCFP-PEN1 and mYFP-VAMP722 were analyzed upon fungal challenge. (A) Schematic drawing depicting the domain structures of the N-terminally tagged SNAP33 and PEN1 proteins.

Figure 23 continued. (B) Co-expression of mYFP-SNAP33 and cCFP-PEN1. Propidium iodide was used to stain fungal structures (upper panel left picture). mYFP fluorescence was excited at 514 nm, cCFP was excited at 405 nm and propidium iodide was excited using a laser of 561 nm. Fluorescence emission was detected at 518 to 578 nm for mYFP, 453 to 500 nm for cCFP, and 600 to 705 nm for propidium iodide. The spore body is indicated by a dashed red line. The round-shaped mYFP signal represents a focal accumulation of mYFP-SNAP33 fusion protein (upper panel central picture) an identical accumulation pattern can be seen for cCFP-PEN1 (upper panel right picture). (B) Lower panel, an overlay of the fluorescence signals obtained for mYFP and cCFP with the transmission picture. cCFP-PEN1 and mYFP-SNAP33 signals completely overlap at sites of fungal attack (white arrowhead) and in non-attacked areas of the plasma membrane. (C) Schematic drawing depicting the domain structures of the N-terminally tagged PEN1 and VAMP722 proteins. (D) Co-expression of mYFP-VAMP722 and cCFP-PEN1. Upper panel, separate images obtained for cCFP and mYFP signals at excitation and emission settings described in (B). mYFP-VAMP722 is shown to focally accumulate beneath attempted *B.g.hordei* entry sites and labels vesicular structures (arrow heads). Lower panel, an overlay of the images obtained for cCFP and mYFP excitation and the transmission channel. Complete overlap of the mYFP and cCFP signal (arrow head), can be seen exclusively underneath the fungal appressorium (AP).

Table 21. Arabidopsis lines expressing fluorochrome-tagged SNARE proteins

Transgene	Genotype*	Localization	F.a. upon *B. g. hordei* challenge
pPEN1::mYFP-PEN1	*pen1*-1	plasma-membrane	Yes
p35s::GFP-PEN1	*pen1*-1	plasma-membrane	Yes **
p35s::cCFP-PEN1	*pen1*-1	plasma-membrane	Yes §
p35s::mYFP-SNAP33	*snp33*-1	plasma-membrane	Yes
p35s::mYFP-VAMP722	*vmp722*-1	plasma-membrane and vesicular structures	Yes $
p35s::cCFP-PEN1/ p35s::mYFP-SNAP33	n.d.	same as single color lines	Yes
p35s::cCFP-PEN1/ p35s::mYFP-VAMP722	n.d.	same as single color lines	Yes

Arabidopsis lines expressing cCFP-PEN1 and mYFP-SNAP33 or cCFP-PEN1 and mYFP-VAMP722 were derived from crosses of the respective single color lines. Resulting F1 and F2 progeny expressing both fluorochromes were analyzed microscopically for subcellular localization of the fusion proteins. n.d., not determined; f.a., focal accumulation underneath fungal appressoria; *, all in Col-0 background; **, see Collins et al. 2003; §, data not shown, and Assaad et al. 2004; $, C.Neu personal communication.

The *Arabidopsis* lines expressing cCFP-PEN1 and mYFP-SNAP33 or cCFP-PEN1 and mYFP-VAMP722 were grown for ten days in a protected environment before spore inoculation with *B. g. hordei*. Confocal images were taken between 16 and 20 hpi (0). Confocal imaging showed that all three SNARE proteins became concentrated at the sites of fungal attack. Focal accumulations appeared as early as 12 hpi (data not shown and C. Neu, personal communication; Assaad et al. 2004, Bhat et al 2005) and seemed to be coordinated in time and space with powdery mildew-induced de-novo cell wall biosynthesis ("papilla formation"; Assaad et al. 2004 and this study). No differences could be observed in the timing or the spatial distribution of cCFP-PEN1 and mYFP-SNAP33 focal accumulations. Both proteins were associated with the plasma membrane and accumulate beneath attempted *B. g. hordei* entry sites.

In contrast to PEN1 and SNAP33, fluorochrome-tagged VAMP722 appeared to localize predominately to mobile intracellular vesicle-like structures (0D arrow heads and C. Neu, personal communication). This is consistent with the expected localization of VAMP (vesicle-associated membrane protein) protein family members. Although mYFP-VAMP722 fluorescent signal did not label the plasma membrane significantly, it was also concentrated to the site of fungal attack and co-accumulates with PEN1 and SNAP33 underneath powdery mildew appressoria (0D).

III.4.4 Focal accumulations appear as plasma membrane independent structures and co-localize with papillae

In previous studies, conflicting data were published on the subcellular localization of the focal accumulation (Assaad et al. 2004, Bhat et al. 2005). Assaad and colleagues found that in *pen1*-1 mutant plants, powdery mildew-induced papilla formation is significantly delayed in comparison to wild-type plants. In addition, these authors showed that fluorochrome-tagged PEN1 concentrated not only beneath attempted fungal entry sites but also accumulated within the interior of the papilla structure observed in confocal cross-sections (Assaad et al. 2004). In contrast, Bhat et al. reported that PEN1 accumulation is associated with the plasma membrane as in plasmolysis experiments lack of focal accumulation at the cell wall was detected. Bhat et al. therefore concluded that the focal accumulation is indicative of fungus-induced lipid micro-domains reminiscent of animal lipid rafts (Bhat et al. 2005). To clarify whether the focal accumulation of PEN1 protein partners resembles lipid micro-domains or whether there

is an association of the fluorescent signal with papillae, I performed plasmolysis experiments using transgenic lines expressing either mYFP-SNAP33, or GFP-PEN1 (Figure 24), or lines co-expressing cCFP-PEN1 and VAMP722 (data not shown). A retraction of the fluorescent signal of the focal accumulation from the cell wall with ongoing plasmolysis was undetectable. In contrast, the focal accumulation seemed to adhere to the cell wall in all inspected transgenic *Arabidopsis* lines. No difference was observed among the individual SNARE proteins (mYFP-SNAP33, GFP-PEN1, mYFP-VAMP722). Leaf epidermal protoplasts often did not shrink completely and the plasma membrane remained attached to the cell wall around focal accumulation sites, which may be caused by the applied plasmolyticum (5 M sorbitol). In the few cases, where the plasma membrane completely detached from the cell wall at sites of focal accumulation sites, the fluorescent signal retained its position (Figure 24A and B). A time lapse series during plasmolysis of *Arabidopsis* leaf epidermal cells expressing mYFP-SNAP33 is shown in Figure 24. With ongoing plasmolysis (from left to right) the plasma-membrane retracted from the cell wall (arrow head) but the focal accumulation retained its position. Similarly, the micrograph of GFP-PEN1 expressing leaf epidermal cells showed a focal accumulation (arrow head) associated with the paramural space after retraction of the plasma membrane in this area (Figure 24B). No specimens were found that showed evidence for GFP-PEN1 or mYFP-SNAP33 containing plasma-membrane micro-domains. My observations are consistent with the finding that GFP-PEN1 fluorescence signals associate with papillae in confocal cross-sections (Assaad et al. 2004).

It is conceivable that the association of fluorochrome-tagged PEN1 with cell wall appositions is a result of secretion to the paramural space (Figure 24C left side) and/or reflects tethering of plasma membrane constituents including PEN1 to the newly synthesized cell wall material at the entry sites (Figure 24C right side). In the latter case, thin, Hechtian strand-like plasma membrane connections could be formed, allowing for a lipid continuum between the periphery of cell wall appositions and the protoplast (Figure 24C right side, HS).

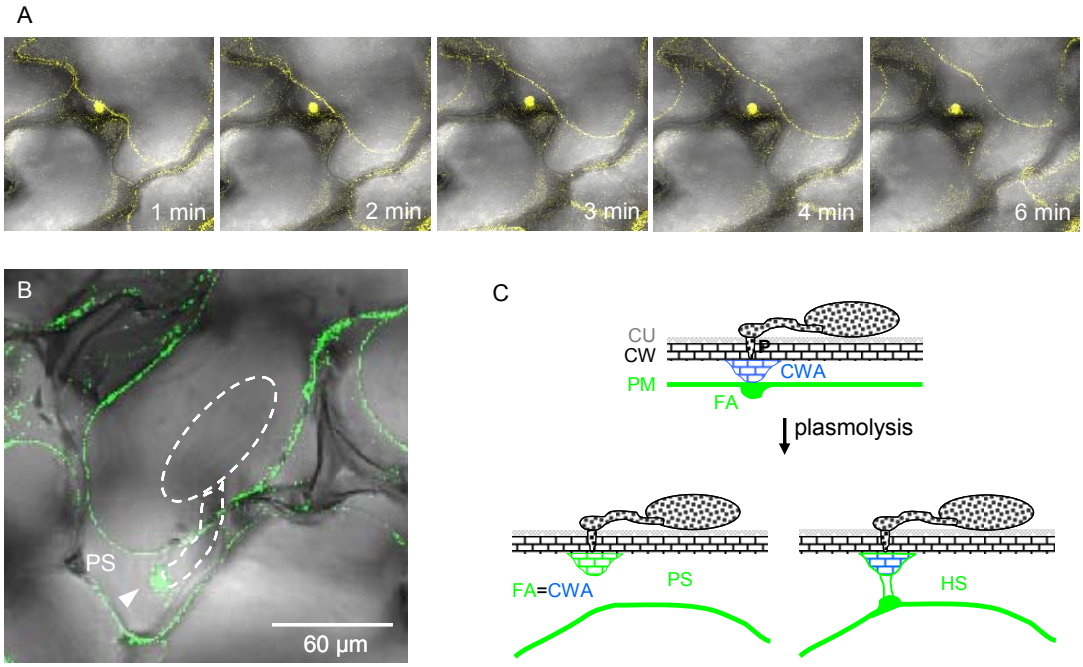


Figure 24. SNARE protein focal accumulations do not retract from the plasma membrane upon plasmolysis. (A) Confocal images of mYFP-SNAP33 expressing plants were taken 16 h after *B. g. hordei* challenge. To induce plasmolysis the leaves were mounted in 5 M sorbitol and imaged immediately. Time point after sorbitol treatment is indicated below the picture. Images were taken at 514 nm to excite mYFP. (B) GFP-PEN1 expressing plants were imaged 12 hpi *B. g. hordei* using a laser of 488 nm for GFP excitation. Plasmolysis was induced as mentioned in (A). Images of plasmolysed epidermal cells were taken 15 min to 30 min after treatment. The *B. g. hordei* spore is indicated by the white dotted line. The focal accumulation (arrow head) of GFP-PEN1 appears to be trapped in the paramural space, a result of plasmolysis-induced separation of plasma membrane and cell wall. (C) Cartoon illustrating two possibilities of SNARE signal retention after plasmolysis. Left picture, exosomal secretion delivers GFP-PEN1 into the paramural space to associate with cell wall appositions. Fluorochrome signals derived from focally accumulating SNARE proteins will also label cell wall appositions. Right picture, the plasma membrane does not disconnect from the cell wall entirely. Residual plasma membrane threads, so-called Hechtian strands, keep the plasma membrane attached to the cell wall apposition thereby retaining signals derived from plasma membrane resident SNARE proteins at the cell wall apposition. PS, paramural space; CU, cutin; CW, cell wall; FA, focal accumulation; CWA, cell wall apposition; HS, Hechtian strands.

However, in microscopic cross sections the fluorescence signal appeared within the complete interior of the papillar structure and not only at the papilla margins (data not shown and D.Meyer personal communication).

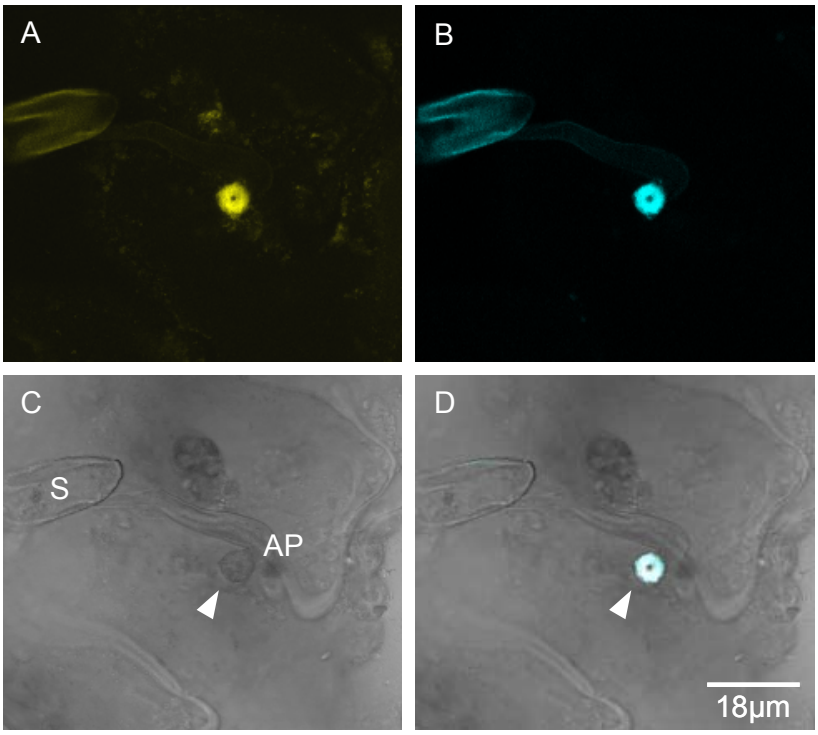


Figure 25. PEN1 focal accumulation colocalizes with callose. Confocal images of ten days-old *Arabidopsis* plants expressing p35S::mYFP-PEN1 were taken at 18 hpi with *B. g. hordei* conidiospore. Leaves were stained in aniline blue staining solution (0,01% aniline blue) for 30 min prior to imaging. (A) YFP excitation at 514 nm. A typical doughnut-shaped mYFP-PEN1 fluorescence signal is shown at *B. g. hordei* attempted entry sites. (B) Aniline blue excitation at 405 nm. Fluorescence emission was measured at 410 to 480 nm. A ring-shaped callose deposition is shown at the attempted *B. g. hordei* entry site. (C) A germinated *B. g. hordei* spore with an appressorium is seen on the leaf surface in the transmission channel. The arrow head points to a round-shaped cell wall apposition underneath the fungal appressorium. (D) Overlay of images obtained using YFP and aniline blue excitation as well as the transmission channel. Note the exact colocalization of the mYFP-PEN1 and the aniline blue signal.

To clarify whether PEN1 might be secreted into the paramural space, I tested if PEN1 focal accumulations would co-localize with papilla. Therefore I used aniline blue staining to visualize papillary callose at 18 hours after *B. g. hordei* conidiospore

inoculation in live *Arabidopsis* epidermal leaf tissue expressing mYFP-PEN1 (Figure 25, Jacobs et al. 2003, Nishimura et al. 2003). At the resolution of the confocal microscope the signal derived from papillary callose and mYFP-PEN1 signal clearly co-localized underneath the fungal appressorium (Figure 25D). Interestingly, electron-micrographic pictures of cell wall appositions in wild-type plants show inclusions of membranous particles in cell wall appositions at powdery mildew entry sites supporting an exosomal-like delivery of membranous or vesicle-like structures to the paramural space (An et al. 2006, Assaad et al. 2004). Thus, it is possible that at least a pool of plasma membrane associated SNARE proteins, including PEN1, is secreted and become trapped in the paramural cell wall appositions during fungal pathogenesis. This unusual process could be conceptually similar to exosome secretion in animals (de Gassart et al. 2004, Keller et al. 2006, van Niel et al. 2006).

IV Discussion

IV.1 Structure function analysis of PEN1

IV.1.1 Phosphoryation at N-terminal residues: A conserved mechanism in syntaxin regulation?

The N-terminus of syntaxin comprising its regulatory helical bundle H_{abc} is a structurally flexible region as shown from multiple structural analyses *in vitro,* including NMR- spectroscopy of mammalian syntaxin 1a (Dulubova et al. 1999), X-ray diffraction crystal structure analysis of squid (*Logilo palei*) neuronal syntaxin (Bracher et al. 2002, Bracher and Weissenhorn 2004), single molecule Förster resonance energy transfer experiments (Margittai et al. 2003) and electron paramagnetic resonance analysis of mammalian syntaxin 1a (Margittai et al. 2001). Thereby, the regulatory helical bundle H_{abc} has been reported to undergo major conformational changes upon binary and ternary complex formation (Margittai et al. 2001, Margittai et al. 2003).
Interestingly, phosphorylation of syntaxins was exclusively detected at single serine residues adjacent to this flexible H_{abc} domain (see alignment in Figure 7), to date. By using an antiserum specific for syntaxin 1 phosphorylated at serine 14, Foletti and co-workers demonstrated that serine 14 of both isoforms of *Rn* syntaxin 1, syntaxin 1a and 1b, in the rat brain is phosphorylated throughout brain development (Foletti et al. 2000). Serine 188 which is located in the linker region between the H_{abc} domain and the SNARE domain of rat syntaxin 1a, was shown to be phosphorylated in a Ca^{2+}-dependent manner *in vitro* and *in vivo* in transfected human embryonic kidney cells (HEKT293T; Tian et al. 2003). Using recombinant and native SNARE proteins from rat brain homogenisate, Risinger and Bennett could demonstrate phosphorylation of rat syntaxin 1a, rat syntaxin 3a and rat syntaxin 4 at serine and/or threonine residues mapping to the N-terminus of the proteins *in vitro* (Risinger and Bennett 1999). In immunoprecipitation experiments in *Saccharomyces cerevisiae,* Sso1p syntaxin phosphorylated at serine 79 was demonstrated to display decreased affinity to the yeast SNAP25 family member protein Sec9 (Marash and Gerst 2001). Similarly, functional SNARE complex formation of the yeast t-SNAREs Tlg1 and Tlg2 was shown to decrease in phosphorylated compared to dephosphorylated proteins *in vitro* and *in vivo*

(Gurunathan et al. 2002). From these analyses it has been speculated that phosphorylation at N-terminal residues affects the conformational state of the regulatory H_{abc} bundle either by weakening the inactive closed conformation (Snyder et al. 2006) or stabilizing by it (Gerst 2003).

It is possible that plant syntaxins follow a similar regulatory mechanism, since N-terminal serine or threonine residues at both, position six and eight are conserved among the SYP1 sub-clade of *Arabidopsis* syntaxins (see alignment in Figure 7). Indeed, rapid phosphorylation of PEN1 and SYP122 syntaxins in response to challenge with the flg22-peptide derived from bacterial flagellin (Felix et al. 1999) has previously been reported in *Arabidopsis* cultured cells (Benschop et al. 2007, Nuhse et al. 2003). In addition, *Nt*SYP121 was demonstrated to be phosphorylated at an unknown residue in an *Avr9/Cf9* race-specific signaling pathway in transgenic tobacco plants expressing the tomato *Cladosporium fulvum Cf9* resistance gene (Heese et al. 2005). However, potential biological functions of these phosphorylation events have not been identified, to date.

Here, I tested whether PEN1 phosphorylation at N-terminal residues may play a functional role in disease resistance responses at the cell periphery, by analyzing phospho-mimic ($PEN1^{S6D, S7D, S8D}$) and phospho-knockout ($PEN1^{S6A, S7A, S8A}$) variants of PEN1 *in planta*. Interestingly, $PEN1^{S6D, S7D, S8D}$ and $PEN1^{S6A, S7A, S8A}$ variants revealed contrasting results. Aspartate phospho-mimic variants, although carrying three highly charged residues (S6D, S7D, S8D), still complemented the *pen1*-1 phenotype, while serine to alanine exchange variants (S6A, S7A, S8A, phospho-knockout variants) were significantly impaired in mediating resistance responses (Figure 10). Three tested $PEN1^{S6D, S7D, S8D}$ lines supported entry rates of *B. g. hordei* ranging from 18 (+/-2) % to 21 (+/-3) % while the two tested $PEN1^{S6A, S7A, S8A}$ lines supported 31 (+/-2) % and 46 (+/-5) % of powdery mildew ingress into leaf epidermal cells, respectively (Figure 10). Thus, *B. g. hordei* conidiospores were almost twice as successful in entering *Arabidopsis* leaf epidermal cells expressing a non-phosphorylatable form of PEN1 compared to cells which expressed a PEN1 phospho-mimic variant. The enhanced fungal entry rates of the non-phosphorylatable $PEN1^{S6A, S7A, S8A}$ variant compare well with the partially non-functional *pen1*-3 allele which supported 39 (+/-4) % of *B. g. hordei* entry (see Figure 9).

Interestingly, restriction of fungal entry were restricted even more by PEN1$^{S6D, S7D, S8D}$ than by the wild-type PEN1 construct, ranging from 18 (+/-2) % to 21 (+/-3) %, compared to 20 (+/-3) % to 30 (+/-5) % for the wild-type construct. The finding that replacement of three N-terminal hydrophilic serines in PEN1 by hydrophobic alanines, but not substitutions by acidic aspartate residues, results in partial loss of PEN1 activity in disease resistance, is consistent with the interpretation of phosphorylation-dependent activity changes of PEN1 rather than activity changes resulting from a general change in PEN1 folding/conformation. Thus, phosphorylation at N-terminal serine residues, which were mimicked by aspartates, appears to be required for full PEN1 activity in non-host resistance to *B. g. hordei*, while de-phosphorylation may not play a role.

However, the non-phosphorylatable PEN1^{S7G} variant also rescued the *pen1*-1 phenotype (Figure 10), indicating that serine 7 and a potential phosphorylation at this residue is dispensable for full PEN1 activity in response to *B. g. hordei*. Phosphorylation of PEN1 at serine 7 has been reported in *Arabidopsis* cultured cells upon elicitation with the pathogen associated molecular pattern (PAMP) peptide flg22 derived from bacterial flagellin but not in response to the fungal PAMP elicitor xylanase from *Trichoderma viride* (Benschop et al. 2007). Although responses to the bacterial PAMPs flg22 and EF-Tu and to oomycete NEP-like elicitor proteins have been shown to activate and suppress an overlapping set of genes and suggested that PAMP triggered downstream signaling may be highly convergent (Qutob et al. 2006, Zipfel et al. 2006, Zipfel et al. 2004), a differential phosphorylation of PEN1 in response to the pathogen derived elicitors flg22 and xylanase, respectively (Benschop et al. 2007), might point to the existence of divergent PAMP-triggered signaling pathways.

Interestingly, *pen1*-1 mutant plants show an enhanced penetration phenotype in response to non-adapted powdery mildews, i.e. *Erysiphe pisi* and *B. g. hordei* (Collins et al. 2003, Lipka et al. 2005). In response to other tested non-adapted pathogens including the oomycete *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* pv *tomato*, *pen1*-1 mutant plants do not support enhanced pathogen entry (Zhang et al. 2007; V. Lipka, unpublished). Thus, lack of differential infection phenotypes with non-adapted, non-powdery mildew pathogens on wild-type and *pen1*-1 mutant plants may indicate efficient secretion of antimicrobial compounds by a PEN1-independent pathway, or alternatively, a resistance mechanism independent of SNARE- based secretion may be operating against these parasites.

Constitutive phosphorylation at N-terminal serine residues of syntaxin 1 in the rat brain is thought to play a role in the selective distribution of this neuronal syntaxin along the axonal membrane (Foletti et al. 2000). Similarly, a pool of PEN1 might be constitutively phosphorylated at N-terminal serine residues to selectively distribute a number of fusion competent PEN1 proteins along the surface-exposed plasma membrane of epidermal cells. Such a pool could be important for rapid stimulus-dependent vesicle fusion reactions e.g. in case of pathogen attack. However, a constitutively phosphorylated form of PEN1 was not detected in a recent study (Benschop et al. 2007). In addition, we failed to identify a phosphorylated form of PEN1 from powdery mildew challenged and/or unchallenged leaf tissue using immunodetection by the PEN1-antiserum (data not shown and C. Kwon, personal communication).

Alternatively, a potentially phosphorylated form of PEN1 might only be transiently induced in powdery mildew attacked cells or might only be present in specific cells, i.e. leaf epidermal cells, or specific subcellular compartments, e.g. distinct regions in the plasma membrane, and therefore represent a small percentage of total PEN1 present in *Arabidopsis* leaf tissue. Immunodetection of PEN1 in total leaf extract may therefore not be sensitive enough to visualize a phosphorylated form of PEN1.

IV.1.2 Amino acid residues in the conserved SNARE domain and at adjacent positions are required for full PEN1 activity.

To examine PEN1 structure-function relationships and to assess potentially shared animal and plant syntaxin functions, I generated *in vitro* and analyzed a number of PEN1 variants in transgenic *Arabidopsis* lines carrying amino acid substitutions previously characterized in studies with animal syntaxins, i.e. syntaxin 1a from *Drosophila melanogaster,* and *Rattus norvegicus,* and Unc-63 syntaxin from *Caenorhabditis elegans,* (Dulubova et al. 1999, Fergestad et al. 2001, Wu et al. 1999; see Table 16 and 18). Each of these amino acid substitutions altering *in vitro* and/or *in vivo* activities of animal syntaxins, resulted in impaired PEN1 activity in plant immune responses to *B. g. hordei* ranging from 29 (+/-8) % to 60 (+/-8) % in comparison to 72 (+/-2) % in *pen1*-1 null mutant plants (Figure 11).

The PEN1$^{L185A, D186A}$ variant displayed strongly impaired PEN1 resistance activity to *B. g. hordei* , i.e. 40 (+/-8) % and 60 (+/-8) % of fungal entry in two tested independent lines. These mutations have been reported to arrest mammalian syntaxin 1a in its open conformation *in vitro* by nuclear magnetic resonance (NMR)-spectroscopy (Dulubova et al. 1999). The biological relevance of these residues was shown by expression of an equivalent *C. elegans* syntaxin in the syntaxin null mutant or unc13-deficient worms (Richmond et al. 2001). Both, wild type sequence and the open conformation variant of the *C. elegans* syntaxin, were able to rescue paralysis and developmental arrest associated with the loss of syntaxin in transgenic worms (Richmond et al. 2001). Interestingly, the open conformation variant but not the wild-type syntaxin could rescue behavioural phenotypes of *C. elegans* mutants lacking a functional copy of the *Unc13* SNARE regulator and was able to partially restore synaptic vesicle fusion events in transgenic worms (Richmond et al. 2001). Thus, in contrast to plant resistance responses, where a putative open conformation PEN1 syntaxin variant failed to rescue the syntaxin null mutant, in worms the open syntaxin variant was functional and in addition, could complement for the loss of an essential accessory SNARE regulator, Unc13 (Aravamudan et al. 1999, Augustin et al. 1999, Brose et al. 2000, Richmond et al. 2001). Unc13, also called Munc13, is a large scaffold protein (>1000 amino acids) which has been proposed to be involved in the transition of closed to open conformation of syntaxins at the synapse in invertebrates and mammals (Aravamudan et al. 1999, Augustin et al. 1999, Betz et al. 1997, Brose et al. 1995, Brose et al. 2000, Sudhof 2004), and is absent in plant genomes. Assuming that the amino acid substitutions L185A, D186A arrest PEN1 in an open conformation as it has been reported for mammalian syntaxin 1a (Dulubova et al. 1999), it is conceivable that plants have evolved other, Unc13-independent, regulatory mechanisms for syntaxin activity.

Similarly, a I236A variant of Drosophila syntaxin 1a, which was strongly reduced in its interaction with the Drosophila accessory Munc18 family protein ROP *in vitro*, retained its ability to form SDS-resistant ternary SNARE complexes *in vitro* and to mediate neurotransmitter release *in vivo,* i.e. in transgenic fly embryos expressing a genomic construct of the syntaxin variant in a null mutant background (Wu et al. 1999). These findings suggested that the interaction of syntaxin with Munc18/ROP in *Drosophila* is inhibitory for secretion (Wu et al. 1999). Here, the corresponding mutation I255A in PEN1 leads to a severe reduction in PEN1 activity. Nevertheless, SM-like proteins are

present in plants in similar numbers as in animals (Pratelli et al. 2004, Sanderfoot et al. 2000, Sutter et al. 2006), which would support the idea that a subset of syntaxin regulatory mechanisms are conserved between plants and animals.

The mammalian syntaxin 1a variant L205A, E206A (corresponding to the PEN1$^{I227A, E228A}$ variant) was described to alter binary complex formation *in vitro* (Dulubova et al. 1999). These amino acid exchanges alter residues reported to be in direct contact with SNAP25 in the crystal structure of the SNARE core complex (Sutton et al. 1998). The activity of this variant to mediate vesicle fusion *in vivo* has not been tested in the original study (Dulubova et al. 1999). The PEN1$^{I227A, E228A}$ variant is partially impaired in mediating *in planta* disease resistance responses (Figure 11). Among the three independent transformants tested, one line, PEN1$^{I227A, E228A}$ #1, exhibited a strong increase in *B. g. hordei* entry supporting entry rates of 50 (+/-5) % compared to 20 (+/-2) % in plants expressing the PEN1 #2 wild-type construct, while two other lines, PEN1$^{I227A, E228A}$ #3 and #5, showed a moderate increase, 36 (+/-4) % and 31 (+/-6) %, comparable to the partially non-functional *PEN1*-3 allele, which supported 39 (+/-4) % of fungal entry (see Figure 11). Statistically, there was no significant difference between plants carrying the partially non-functional allele *PEN1*-3 and all three independent transformants ($P > 0,05$) but also no significant differences to wild-type Col-0 plants were observed, except for PEN1$^{I227A, E228A}$ #1 ($P = 0,0002$). Further analysis assessing more individuals of line #5, of which only four individuals were tested to date, and additional independent transgenic lines will be useful to substantiate my observations with the PEN1$^{I227A, E228A}$ construct.

Similarly, only few individuals of transgenic lines expressing the PEN1$^{A262V, I266A}$ variant could be analyzed so far (see Results section III.1.4). Nevertheless, the few transgenic individuals tested from three independent transgenic lines suggest a functional impairment also for the PEN1$^{A262V, I266A}$ variant (Figure 11). Transgenic fly embryos transformed with a genomic syntaxin construct carrying the corresponding mutations (A243V, V247A) displayed pronounced reduction in neurotransmission but were able to secrete cuticle, indicating that non-neuronal secretion was unaffected (Fergestad et al. 2001). The currently available data on the PEN1$^{A262V, I266A}$ variant needs to be validated in future experiments including additional independent transgenic lines. The amino acids analyzed here might be an example for functional conservation between animal and plants syntaxins since they might be required for both, full activity

of a *Drosophila* syntaxin in synaptic transmission and full activity of PEN1 syntaxin during plant defence responses.

Collectively, my data provides for the first time functional evidence that secretory syntaxins in plants are subject to phospho-regulation at N-terminal residues and raises the question regarding potential kinases and phosphatases involved in these processes.
In animals, *Rn* syntaxin 1a has been shown to be an *in vitro* substrate of casein kinase I (CKI) and CKII (Dubois et al. 2002, Hirling and Scheller 1996, Risinger and Bennett 1999) and was found to phosphorylated at serine 14, a predicted CKII phosphorylation site (Foletti et al. 2000). Furthermore syntaxin 1a has been suggested to be a substrate for the calcium-dependent death associated protein kinase (DAPK) by *in vitro* kinase assays and colocalization studies and immunoprecipitation experiments *in vivo* (Tian et al. 2003). In plants, kinases have been shown to act at multiple steps in resistance responses. Receptor-like kinases (RLK) including the *Arabidopsis* FLS2 required for flg22-preception (Gomez-Gomez and Boller 2000), EFR, required for sensing the bacterial elongation factor Ef-Tu (Zipfel et al. 2006) and Xa21 protein kinase conferring race-specific resistance to *Xanthomonas oryzae* pv *oryzae* in rice (Song et al. 1995) are involved in the recognition of pathogen-derived elicitors, while MAP (mitogen-activated protein) kinase cascades (Asai et al. 2002, Daxberger et al. 2007, Nurnberger and Scheel 2001) and Ca^{2+}-depedent kinases were shown to be involved in downstream signalling in plant disease resistance responses to bacterial, fungal and oomycete pathogens (Nurnberger and Scheel 2001, Romeis 2001, Romeis T et al. 2001). To identify enzymes involved in syntaxin phosphorylation among the numerous plant Ser/Thr kinases active in plant defences will be a challenge for future studies.

Substitutions in any of the tested conserved amino acids in or adjacent to the PEN1 SNARE domain appeared to interfere with PEN1 activity in disease resistance responses, supporting the hypothesis that PEN1 functions through SNARE domain-dependent interactions in ternary SNARE complexes. This supports the finding that purified PEN1 and SNAP33 proteins engage in ternary SDS-resistant SNARE complexes with *in vitro* with VAMP7 protein family members and is consistent with the recent identification of two *VAMP* genes, *VAMP721* and *VAMP722* of *Arabidopsis*, which restrict entry of *B. g. hordei* similar to *PEN1 in planta* (Kwon et al. in preparation). In transgenic *Drosophila* and *C. elegans*, the respective I255A and L185A,

D186A substitutions in syntaxin 1a, which have been reported to impair binding of regulatory SM proteins however, did not result in a loss of function (Richmond et al. 2001, Wu et al. 1999). This supports the idea, that plants may have evolved additional ways of regulating their surplus of components of the SNARE-based vesicle trafficking machinery (Bock et al. 2001, Sanderfoot 2007, Sanderfoot and Raikhel 2003).

IV.2 Functional diversification and redundancy of PEN1 and SYP122 syntaxins

Among the 18 syntaxin encoding genes in the *Arabidopsis* genome, *SYP122* shows the highest sequence similarity to *PEN1* and may be the product of a recent gene duplication (see introduction, section I.4.3). Despite high sequence identity (see alignment in Figure 7) only PEN1 contributes to plant immune responses against powdery mildew parasites, which is indicative of a complete functional diversification between the two proteins in disease resistance. Here, I could show that SYP122 steady state levels are highly pathogen responsive and increase at least ten-fold within the first 24 hrs after *B. g. hordei* challenge. In contrast, pathogen-induced changes of PEN1 abundance are subtle, but occur in the same time range during pathogenesis. The difference in protein abundance correlates well with the differential responsiveness of *SYP122* and *PEN1* at the levels of mRNA accumulation (Assaad et al. 2004). Similarly, the *Arabidopsis* t-SNARE SNAP33 has been shown to be upregulated at both, the mRNA transcript and protein level after pathogen challenge (Wick et al. 2003). Wick et al. hypothesize that a general transcriptional upregulation of SNARE components, as seen for SNAP33, might compensate for the increased secretory activity observed during plant defense responses and may reflect the contribution of SNARE proteins to the export of pathogenesis-related (PR) proteins and antimicrobial compounds (Wick et al. 2003). Moreover, SNARE components of the secretory machinery have been identified to be upregulated during immune responses in macrophages of the mammalian immune system (Murray et al. 2005a, Murray et al. 2005b, Pagan et al. 2003, Stow et al. 2006), pointing to the possible existence of shared vesicle-based immune mechanisms in plants and animals.

IV.2.1 Functional diversification of PEN1 and SYP122 is complete in pre-invasion resistance to *B. g. hordei*

Because of the markedly different pathogen-inducible accumulation profiles of PEN1 and SYP122 it is conceivable that the previously reported functional specialization of both proteins merely reflects insufficient SYP122 levels at time points that are critical to restrict *B. g. hordei* ingress (~ 10-15hpi; Figure 15; Thordal-Christensen et al. 2000). However, transgenic plants that constitutively overexpress SYP122 in a *pen1*-1 background at high levels, failed to restrict *B. g. hordei* entry, i.e. SYP122 fails to complement for the loss of PEN1 in immune responses to *B. g. hordei* even upon overexpression (Figure 19). This new observation suggests that functional differences between PEN1 and SYP122 are encoded by differences in their amino acid sequences. In this context, it is of note that the tested syntaxin chimera 2211 (Figure 17) showed severely reduced activity in disease resistance responses to *B. g. hordei*. One interpretation of this result is that the N-terminus of PEN1 comprising the regulatory H_{abc} bundle and parts of the linker region is indispensable for proper PEN1 function. A reciprocal domain swap construct could directly test this hypothesis.

IV.2.2 Other functions of PEN1 and SYP122 syntaxins in disease resistance

Since a delay in timing of papilla formation between *pen1*-1 mutant and wild-type plants was observed following challenge with *B. g. hordei* conidiospores, PEN1 was accounted to be critical for the timely assembly of cell wall appositions (Assaad et al. 2004, Shimada et al. 2006). Lack of a corresponding infection phenotype in *syp122*-1 null mutant plants, and severe dwarfism and leaf necrosis in the absence of the pathogen in *pen1/syp122* double mutants, has been interpreted as evidence for an additional PEN1 function that is shared with SYP122 and may contribute to general secretion (Assaad et al. 2004).

Table 22. List of thirty genes highly co-expressed with *PEN1* and *SYP122* (*P*>0,7)

rank *	avg *P* *	locus	(putative) protein function
1	0.80	At1g07000	exocyst subunit EXO70 family protein
2	0.80	At2g38470	WRKY family transcription factor (WRKY33)
3	0.80	At1g19020	expressed protein
4	0.88	At3g52400	syntaxin, putative (SYP122)
5	0.88	At3g11820	syntaxin 121 (SYP121)
6	0.77	At4g34390	putative extra-large guanine nucleotide binding protein, putative G-protein
7	0.77	At5g13190	expressed protein
8	0.77	At5g25930	leucine-rich repeat family protein / protein kinase family protein
9	0.76	At1g29690	expressed protein
10	0.76	At3g09830	putative protein kinase
11	0.76	At1g05575	expressed protein
12	0.75	At4g20830	FAD-binding domain-containing protein
13	0.76	At5g66210	calcium-dependent protein kinase family protein / CDPK family protein (CPK28)
14	0.75	At1g28380	expressed protein
15	0.74	At4g34150	C2 domain-containing protein
16	0.75	At1g14370	protein kinase (APK2a)
17	0.73	At3g59080	aspartyl protease family protein
18	0.73	At1g18570	myb family transcription factor (MYB51)
19	0.73	At5g25440	protein kinase family protein
20	0.72	At4g33050	calmodulin-binding family protein
21	0.73	At2g37940	expressed protein
22	0.72	At3g45640	putative mitogen-activated protein kinase MAPK (MPK3)
23	0.73	At3g05200	zinc finger (C3HC4-type RING finger) family protein (ATL6)
24	0.72	At5g06320	harpin-induced family protein / NDR1/HIN1-like protein 3 (NHL3)
25	0.72	At1g55450	embryo-abundant protein-related
26	0.72	At2g18690	expressed protein
27	0.71	At5g54490	calcium-binding EF-hand protein, putative (PBP1)
28	0.71	At4g36500	expressed protein
29	0.71	At1g13210	haloacid dehalogenase-like hydrolase family protein
30	0.73	At5g61210	SNAP25 homologous protein SNAP33 (SNAP33)

* to both query loci *PEN1* (At3g11820) and *SYP122* (At3g52400); avg, average; *P*, correlation probability value.

PEN1 and *SYP122* are highly co-expressed with other genes involved in secretory processes, including the exocyst subunit *EXO70* (At1g07000; *P* = 0,8) and *SNAP33* (At5g61210; *P* = 0,73) as well as with the WRKY33 transcription factor (At2g38470; *P* = 0,8) which is required for disease resistance responses against necrotrophic pathogens, i.e. *Alternaria brassicicola* and *Botrytis cinerea* (listed in Table 22; Obayashi et al. 2007, Wan et al. 2004, listed in Table 22; Zheng et al. 2006; M.Humphry, personal communication; ATTED II, http:://www.atted.biotech.ac.jp), suggesting a possible engagement of either syntaxin in defense-associated processes. Noticeably, six protein kinases appear among the first 30 genes co-expressed with both *SYP122* and *PEN1* (Table 22), including two protein kinase family proteins (At5g25930, *P* = 0,77; and At5g25440, *P* = 0,73), the Ser/Thr kinase APK2a (At1g14370, *P* = 0,75; Ito et al. 1997), a putative protein kinase (At3g09830, *P* = 0,76), a calcium-dependent protein kinase family protein (At5g66210, *P* = 0,76) and a putative MAPK (At3g45640, *P* = 0,72). This gives an additional indication for a potential role of protein kinases and phosphorylation in syntaxin-mediated processes in plants and is consistent with the idea that PEN1 activity in disease resistance may be regulated by phosphorylation at N-terminal serine residues. In future experiments these kinases could serve as potential candidates for the enzymes involved in syntaxin phosphorylation.

Since PEN1 was shown to be required for pre-penetration resistance in response to *B. g. hordei* (Collins et al. 2003, Lipka et al. 2005), it is conceivable that SYP122 might play a yet unidentified role in post-penetration resistance to *B. g. hordei*, which is masked or suppressed in the presence of a functional copy of PEN1. In this respect it would be interesting to test if *syp122* mutant plants would display super-susceptibility to adapted powdery mildews at a post-invasion level and to analyze whether SYP122 might be required to restrict secondary penetration events. In addition, SYP122 might contribute to resistance responses to other parasites not tested so far, even if in a recent publication *pen1* and *syp122* single mutants showed wild-type-like responses to virulent *Pseudomonas syringae* pv *tomato* (Zhang et al. 2007).

Differential defensin gene expression in response to the non-host *B. g. hordei* and the adapted *E.cichoracearum* powdery mildew have been demonstrated previously by transcriptional profiling in *Arabidopsis* (Zimmerli et al. 2004). In plants inoculated with the non-host powdery mildew, the authors reported a correlated expression of defensin

genes, which was absent in plants infected with the host pathogen (Zimmerli et al. 2004). *PDF1.2a* defensin gene expression is a commonly used marker for the activation of the jasmonic acid (JA) /ethylene (ET) defense signaling pathway (Penninckx et al. 1998). Interestingly, *pen1/syp122* double mutants but not the single mutants were shown to have elevated transcript levels of the defensin *PDF1.2a* in RT-PCR analyses of unchallenged tissue (Zhang et al. 2007), which is normally correlated with activation of JA/ET signaling in non-host resistance responses. This finding was interpreted as a regulatory role for both syntaxins in this defense signaling pathway (Zhang et al. 2007). An additional function of PEN1 and SYP122 in the regulation of the salicylic acid (SA) defense signaling pathway has been proposed, since SA-levels were elevated in both, *pen1* single and more pronounced, in *pen1/syp122* double mutants. SA-signaling is thought to counteract JA/ET-based signaling (Spoel et al. 2003).

Whether these defense signaling related phenotypes of *pen1/syp122* double mutant plants are caused by a direct function of *PEN1* and *SYP122* gene products or simply reflect secondary effects related to the lack of PEN1 and SYP122-based vesicle trafficking processes, remains to be elucidated. The importance for the secretory machinery in resistance processes is highlighted by the finding that mutations in genes encoding for protein folding and secretory components of the endoplasmatic reticulum (ER) i.e. *BI2P* and *SEC61α*, result in loss of SA-induced PR-1 secretion and systemic acquired resistance responses (Wang et al. 2005). Since combined mutations in *PEN1* and *SYP122* secretory syntaxins result in disturbed SA signaling in the *pen1/syp122* double mutant (Zhang et al. 2007), it is conceivable to speculate that both PEN1 and SYP122 might represent secretory components downstream of ER-located BIP2 and SEC61α and might play a role in SA-induced PR-protein secretion at the plasma membrane, rather than acting as direct regulators of the interconnected SA and JA/ET signaling pathways.

IV.3 An interaction of PEN1 with the resistance regulator MLO2?

PEN1 and several related *Arabidopsis* syntaxins, including PEN1, SYP122 and SYP132, have been shown to interact with MLO2 in yeast (C. Consonni, unpublished). Additionally, the barley orthologs of MLO2 and PEN1, *Hv*Mlo and *Hv*Ror2, were shown to interact in barley leaf epidermal cells using Förster resonance energy transfer

(FRET) measurements of fluorochrome-tagged proteins in a single cell transient expression system (R. Bhat, M. Kwaaitaal, unpublished). Because loss of function mutations in both, barley *HvMlo* and *Arabidopsis MLO2* result in resistance to all tested adapted powdery mildew species (Buschges et al. 1997, Consonni et al. 2006), it has been hypothesized that adapted powdery mildews might target *Hv*Mlo/MLO2 to suppress *Hv*Ror2/PEN1-mediated vesicle trafficking-based plant defenses at the cell periphery (Panstruga 2005, Schulze-Lefert 2004).

Here, I have identified three PEN1 variants which fail to interact or showed a pronounced reduction in the interaction with MLO2 in a yeast two-hybrid assay (Figure 12). All tested variants, which carried exchanges in the N-terminal serine residues of PEN1, PEN1$^{S6D, S7D, S8D}$ and PEN1$^{S6A, S7A, S8A}$, were strongly reduced in the interaction. Single serine to alanine exchanges at the N-terminus and PEN1 variants carrying amino acid substitutions at other positions, i.e. in the SNARE domain, showed no effect, while a substitution of serine 7 to glycine was partially compromised in the interaction with MLO2 (Figure 12). Since both, phospho-mimic and phospho-knockout variants of PEN1 fail to interact with MLO2 in yeast, N-terminal serine residues or overall folding at the N-terminus of PEN1 might influence the interaction with MLO2 in yeast. It would be interesting to see, if loss of interaction would also be detected for *Hv*Mlo and the respective barley *Hv*Ror2 "phospho" variants using FRET analysis.

To test whether loss of interaction with MLO2 in yeast might have any relevance for MLO2-mediated susceptibility to adapted powdery mildews *in planta*, I tested the infection phenotype of transgenic lines carrying PEN1$^{S6D, S7D, S8D}$ or PEN1$^{S6A, S7A, S8A}$ variants with the adapted powdery mildew *E. cichoracearum* with the support of our collaborators at Stanford University (M. Lim, B-H. Hou, S. Somerville). The pre-invasion resistance phenotype of *mlo2* mutant plants to *E.cichoracearum* has been found to be partially suppressed in *mlo2/pen1* double mutants (Consonni et al. 2006; see Figure 14). If loss of interaction between MLO2 and PEN1$^{S6D, S7D, S8D}$ and PEN1$^{S6A, S7A, S8A}$ in the yeast two-hybrid analysis would reflect loss of interaction between MLO2 and the PEN1 variants *in planta,* PEN1$^{S6D, S7D, S8D}$ and PEN1$^{S6A, S7A, S8A}$ variants would be expected to phenocopy *mlo2* single or *mlo2/pen1* double mutant plants. However, this was not the case. Transgenic plants expressing either PEN1$^{S6D, S7D, S8D}$, or PEN1$^{S6A, S7A, S8A}$, supported *E.cichoracearum* growth comparable to wild-type Col-0 plants (Figure 14).

One interpretation of this finding is that N-terminal serine residues may not play a critical role for responses to the adapted *E.cichoracearum* powdery mildew pathogen, while a contribution of these amino acid residues to resistance responses against the non-adapted *B. g. hordei* could be demonstrated (Figure 10 and Figure 14). Alternatively, this heterologous yeast-based interaction assay may not reflect the situation *in planta,* i.e. the detected interaction between bait and prey construct may be mediated by or depend on a yeast adaptor protein, which may be absent *in planta.*
Whether a physical association of MLO proteins with syntaxins, i.e. *Arabidopsis* PEN1 and barley *Hv*Ror2, as suggested from yeast two hybrid and FRET analyses (Figure 12; C.Consonni, R.Bhat, M. Kwaaitaal, unpublished), significantly contributes to syntaxin regulation in plants (Panstruga 2005, Schulze-Lefert 2004), remains to be elucidated.

Interestingly, in the interaction with the non-adapted *B. g. hordei* a significant suppression of the *mlo2* phenotype was seen in *mlo2/pen1* double mutant plants, similar to the previously reported suppression of *mlo2*-based resistance to adapted powdery mildews (Figure 14; Consonni et al. 2006). This provides evidence that *MLO2* and *PEN1* are genetically linked also in resistance responses to the non-adapted *B. g. hordei*. Thus, apart from adapted powdery mildew species, also non-adapted powdery mildews, including *B. g. hordei* might have learned to target MLO proteins for exploiting syntaxin based vesicle trafficking responses at the cell periphery (Panstruga 2005, Schulze-Lefert 2004). However, double mutant analysis revealed that *mlo2*-based pre-invasion resistance is also broken in combination with other penetration mutants identified as deficient in non-host entry in *Arabidopsis* leaf epidermal cells, *pen2* and *pen3*, encoding a gycosyl-hydrolase and an ABC transporter, respectively, (Lipka et al. 2005, Stein et al. 2006). Since these genes, *PEN2* and *PEN3,* are thought to act in a pathway distinct from *PEN1*, *MLO2* might be a general regulator of pre-invasion resistance upstream of *PEN1, PEN2,* and *PEN3*. For this reason, and because *mlo*-based resistance has been demonstrated to be durable and effective against all known powdery mildew isolates (Buschges et al. 1997, Jorgensen 1992) and is independent of SA-and JA/ET- signaling (Consonni et al. 2006), *mlo*-based resistance has been speculated to be mechanistically identical to non-host resistance (Humphry et al. 2006).

Callose-containing cell wall appositions (papillae) are thought to constitute a physical barrier against invading pathogens and reinforce the cell wall at sites of wounding

(Bushnell and Bergquist 1974, Jacobs et al. 2003, Nishimura et al. 2003). When challenged with *B. g. hordei*, *mlo* mutant barley plants show an accelerated onset of papilla formation and spontaneous callose deposition in the absence of the pathogen (Wolter et al. 1993, Zeyen et al. 1993). The latter is also detectable in *mlo2* mutants of *Arabidopsis* (Consonni et al. 2006). In contrast, *Arabidopsis pen1* mutant plants display a delay in papilla formation in response to *B. g hordei* compared to wild-type plants (Assaad et al. 2004). This alteration in timing of papilla formation correlates with enhanced pre-invasion resistance in plants lacking a functional copy of *Mlo* and a decrease in pre-invasion resistance in *pen1* mutants to *B. g. hordei* or *mlo2/pen1* double mutants (Figure 14; Consonni et al. 2006). Surprisingly, *Arabidopsis* plants lacking the enzyme required for the biosynthesis of callose at sites of wounding and pathogen attack, PMR4/GSL5*,* are resistant rather than susceptible to adapted powdery mildews (Jacobs et al. 2003, Nishimura et al. 2003), indicating that callose or callose synthase may have additional roles in pathogenesis. Instead it may be required by some fungi including adapted powdery mildews to establish an infection and may therefore not contribute to pre-invasion disease resistance at the cell periphery.

IV.4 Subcellular localization of SNARE proteins involved in pathogen defense

Using confocal microscopy, I have shown here that all three tested fluorochrome-tagged SNARE proteins, i.e. fluorochrome-tagged PEN1 under the control of both, 35S overexpression promoter (p35S) and native regulatory sequences, and fluorochrome-tagged SNAP33 as well as fluorochrome-tagged VAMP722 both of which driven by p35S, focally accumulate at sites of attempted fungal ingress (see Figure 20 and 0; focal accumulation of VAMP722 was previously reported by C. Neu, unpublished). This is in accordance with a previous publication where a subset of tested barley fluorochrome-tagged plasma membrane proteins became concentrated (e.g. barley MLO and barley ROR2 syntaxin) at attempted sites of *B. g. hordei* ingress in barley leaf epidermal cells (Bhat et al. 2005). However, this study was based on a transient single cell expression system, in which the expression levels of the genes of interest cannot be controlled and may thus lead to ectopic localization of the fusion proteins.

Furthermore, eight fluorochrome-tagged plasma membrane marker proteins that do not have obvious or known roles in plant defense responses, and PEN3-GFP, which is like PEN1, required for pre-invasion resistance to non-adapted powdery mildews in

Arabidopsis (Stein et al. 2006), all were shown to accumulate underneath *Erysiphe cichoracearum* appressoria in transgenic *Arabidopsis* lines (Koh et al. 2005, Stein et al. 2006). Except of functional BRI1-GFP and functional PEN3-GFP under the control of native upstream regulatory sequences (Friedrichsen et al. 2000, Stein et al. 2006), these fluorochrome-tagged proteins include eight random *Arabidopsis* GFP-cDNA fusion lines driven by the 35S overexpression promoter, whose functions or activities were not tested, i.e. a truncated syntaxin VAM3, two low-temperature induced proteins, LTI6a and 6b, three aquaporins, PIP1b, PIP2a, and SIMIP, and one out-of-frame-fusion protein (Cutler et al. 2000), thus the biological relevance of the concentration of theses proteins/protein fragments beneath attempted powdery mildews entry sites is questionable. Except for GFP-BRI1 and PEN3-GFP, an ectopic localization of the overexpressed plasma membrane proteins to sites of fungal attack cannot be excluded.

PEN1 has been proposed to accumulate to higher levels than other PM-proteins implementing a certain degree of specificity behind this accumulation mechanism (Assaad et al. 2004). Supporting this idea, I could demonstrate for the first time that a functional mYFP-PEN1 fusion protein expressed at native protein levels showed an accumulation pattern identical to overexpressed GFP-PEN1 (Figure 20).

Here it is of note that focal accumulation of PEN1 might be limited to interactions with few parasites including powdery mildews, since it is absent from attack sites of other plant pathogens including the hemibiotrophic fungi *Colletotrichum species, C. lagenarium, C. destructivum, C. higginsianum,* and *C. graminicola,* and *Magnaporthe grisea,* as well as the oomycete *Peronospora parasitica* (D. Meyer, personal communication; Shimada et al. 2006). Furthermore, PEN1 accumulation in response to *B. g. hordei* was independent of PEN1 activity since all tested amino acid exchanges rendering PEN1 (partially) non-functional, including PEN1$^{A262V,\ I266A}$, PEN1$^{L185A,\ D186A}$ and the PEN1-3 mutant protein, retained the ability for focal accumulation (data not shown), supporting the idea that the focal accumulation is not a marker for PEN1 activity in pre-invasion resistance and may be independent of PEN1 activity. Interestingly, a similar phenomena of focal clustering of vesicle trafficking components including SNARE proteins is seen at the immunological synapse, the contact site established between the activated T-cell and antigen-presenting cells in the mammalian immune system (Bossi and Griffiths 2005, Das et al. 2004, Huse et al. 2006, Murray et

al. 2005a), where focal secretion of defense-related compounds, i.e. interleukins, is required.

Fluorochrome-tagged PEN1 also co-localized with papillary callose formed beneath fungal appressoria (Figure 25) and PEN1 accumulation in papillary cell wall material remains visible even ten days after inoculation with *B. g. hordei* conidiospores (D. Meyer, personal communication). Assuming absence of significant protein turnover in this paramural structure, this would indicate that at least the fluorochrome maintains proper folding for several days. Since the fluorescence signal of fluorescence proteins from the hydromedusa *Aequorea Victoria* is known to be pH sensitive (Shaner et al. 2005) secreted GFP or mYFP must be protected from detrimental pH changes in the paramural space in order to retain fluorescence. These findings differ from neuronal vesicle trafficking, where cytosolic α-SNAP and NSF ATPases are known to resolve the SNARE complex supercoils to recycle SNARE components (reviewed in Bonifacino and Glick 2004).

However, it is possible that a portion of PEN1-SNAP33-VAMP721/VAMP722 complexes discharge cargo at the plasma membrane and is subsequently recycled. The detection of GFP-PEN1, mYFP-SNAP33 and mYFP-VAMP722 fluorescence at and in a paramural compartment (papilla) that appeared to detach from the plasma membrane upon plasmolysis (Figure 24) may be indicative of a second pool of these SNAREs that are secreted by *B. g. hordei* attacked epidermal cells. In animals, a poorly defined exosomal secretory pathway has been proposed to function in the elimination of obsolete proteins in intact vesicles (de Gassart et al. 2004, Li et al. 2006). Electron micrographs of barley cell wall appositions formed in response to *B. g. hordei* attack contain membrane enclosed vesicles which might point to exosome formation during the formation of this paramural structure (An et al. 2006, Assaad et al. 2004). Future quantitative measurements of fluorochrome-tagged PEN1 wild-type and non-functional variants at attempted *B. g. hordei* entry sites is needed to examine whether the secretion of presumed exosome-like vesicles has any functional relevance to restrict powdery mildew ingress.

Preliminary data obtained with novel *Arabidopsis* ethyl methane sulfonate (EMS) derived mutants that show either enhanced or reduced GFP-PEN1 accumulation at sites of attempted *B. g. hordei* entry do not support a direct relationship between focal GFP-PEN1 accumulation levels and PEN1 activity in disease resistance. The respective

mutants retain wild-type-like *B. g. hordei* entry rates (D. Meyer, personal communication). This illustrates the difficulties in the interpretation of cell biological phenomena despite the application of fluorochrome-tagged marker proteins.

Lipid-like entities and membranous structures have frequently been found in papillae supporting the idea that exosomal secretion might be involved in plant immune responses at the plasma membrane (Aist 1976, An et al. 2006, Assaad et al. 2004, Bushnell and Bergquist 1974, Mims et al. 2000, Zeyen and Bushnell 1979). Since massive plasma membrane rearrangements and focal delivery of vesicles and vesicle-like structures to the plasma membrane occur during pathogen attack (An et al. 2006, Schmelzer 2002, Zeyen and Bushnell 1979), the deposition or compaction of vesicle-like structures in paramural cell wall appositions could be interpreted as a mechanism to eliminate obsolete membrane material required for maintaining membrane homeostasis (An et al. 2006).

Alternatively, membranes associated with SNARE protein complexes could be utilized by the fungus to increase host membrane surface area required for the formation of the extrahaustorial membrane (EHM; Koh et al. 2005, Schulze-Lefert 2004). The origin of the EHM is still unclear (Green 2002). In a recent study eight tested fluorochrome-tagged plasma membrane marker proteins (see above) failed to label to the EHM of *E.chichoracearum*, indicating that the EHM may not be continuous with the plasma membrane (Koh et al. 2005), unlike it has been inferred from early electron microscopic analysis (Littlefield and Bracker 1970). Since lateral plasma membrane protein movement to the EHM seems to be limited by the haustorial neck, i.e. the tested plasma membrane marker proteins were seen at a collar like shape surrounding the haustorial neck, but not in the EHM, Koh et al. conclude that the haustorial neck represents a seal between the plasma membrane or EHM and the fungal cell wall (Bushnell 1972, Green 2002). Consequently, the authors propose that (i) the EHM either forms by the invagination of the host plasma membrane and subsequent differentiation whereby the haustorial neck might act as a molecular sieve for protein sorting, or (ii) that specialized host vesicles contribute to the formation of the EHM (Koh et al. 2005, Panstruga 2005, Schulze-Lefert 2004). During phagocytosis in innate immune responses in animals, similar increases in plasma membrane surface area are required (Stow et al. 2006). In activated macrophages, phagocytotic cells of the mammalian immune system, SNARE mediated fusion events of vesicles derived from ER, lysosomes, and endosomes

contribute to the increase in the surface area (Murray et al. 2005a, Stow et al. 2006), supporting the latter model for the formation of the EHM (Koh et al. 2005).

IV.5 A model for PEN1 function in cell wall-associated disease resistance

Several lines of evidence suggest that PEN1 may be part of a secretory vesicle trafficking machinery mediating pre-invasion resistance at the cell periphery (illustrated in Figure 26). These evidence include the detection of a SDS-resistant PEN1 and SNAP33 containing high molecular weight complex, reminiscent of a ternary SNARE complex, from *B. g. hordei* challenged *Arabidopsis* leaf tissue (Kwon et al. in preparation), and the recent finding that transgenic *Arabidopsis* plants depleted in *VAMP721/722* transcripts showing a penetration phenotype similar to *pen1*-1 mutant plants (Kwon et al. in preparation). A SNAP25-like gene of barely, *HvSnap34* was demonstrated to be required for full *mlo*-mediated penetration resistance by transient single cell gene silencing experiments (Collins et al. 2003, Douchkov et al. 2005)
Furthermore, novel data documented here showed that conserved amino acid residues in the SNARE domain of PEN1 are required for full PEN1 activity against *B. g. hordei* in transgenic *Arabidopsis* plants (Figure 11). The cargo of presumed PEN1-directed vesicles might include cell wall material and antimicrobial compounds such as pathogenesis related PR-1 protein to stop *B. g. hordei* invasion. Additionally, PEN1 activity in pre-invasion resistance might be modulated via N-terminal phosphorylation, since phospho-mimic but not phospho-knockout variants of PEN1 retained activity in resistance responses to *B. g. hordei* (Figure 10).

A second aspect of PEN1 activity may involve the timely assembly of papilla in response to *B. g. hordei* (Assaad et al. 2004). Since *Arabidopsis pen1*-1 mutants upon *B. g. hordei* challenge retained the ability to form papillae, although with a significant delay compared to wild-type plants, it is conceivable that other syntaxins control the transport of vesicles containing e.g. general cell wall material, to complete papilla formation after the initial PEN1-mediated phase (Assaad et al. 2004). Fourier transform infrared spectroscopy for principal component analysis of the plant cell wall (Chen et al. 1998) revealed a primary cell wall defect in three week-old *syp122* null mutant plants (Assaad et al. 2004).

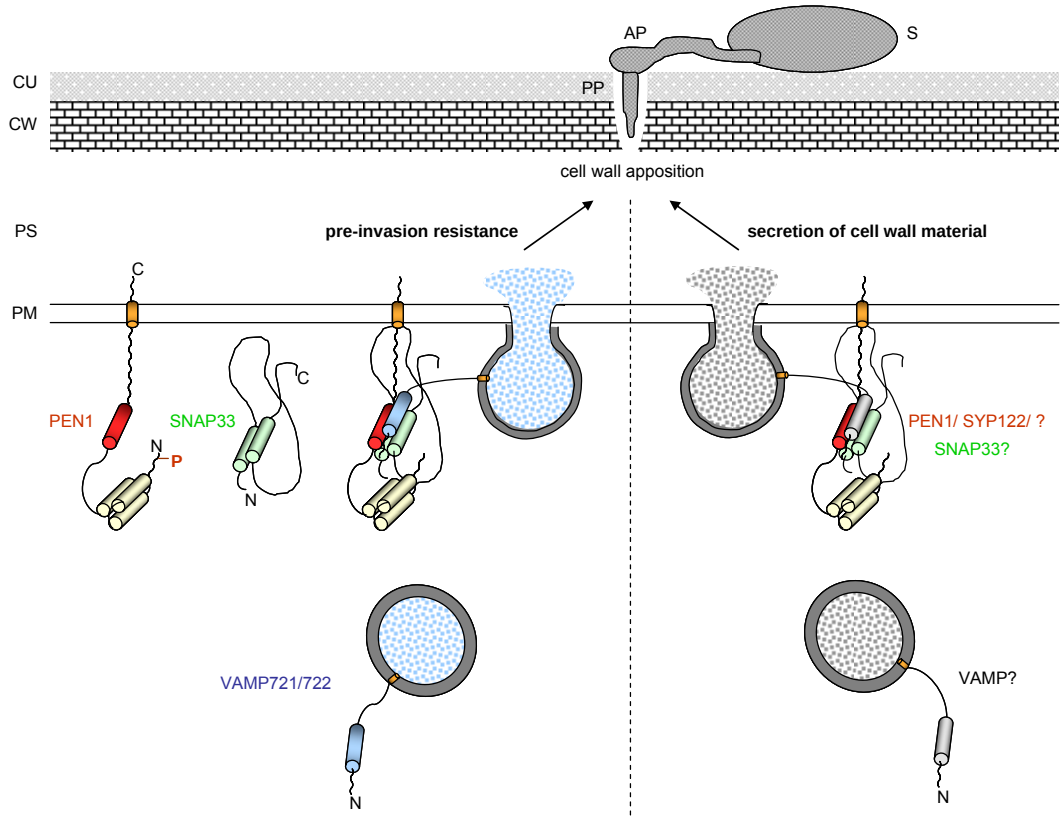


Figure 26. A model for a dual role of PEN1 in pre-invasion resistance and secretion of papillary cell wall material. Schematic drawing depicting the domain structures of PEN1, SNAP33 and VAMP721/722 at the plasma membrane of an epidermal leaf cell of Arabidopsis attacked by a *B. g. hordei* conidiospore (S). PEN1-containing SNARE-complexes contribute to at least two processes at the cell periphery: (i) A PEN1, SNAP33 and VAMP721/722 containing complex mediates pre-invasion resistance to *B. g. hordei*. PEN1 syntaxin activity may be regulated by phosphorylation (P in red color, left side). (ii) PEN1 and SYP122, both contribute to the general secretion of cell wall material to the paramural space. Other components of presumed PEN1 or SYP122 SNARE complexes are currently unknown. Additional syntaxins and corresponding SNARE complexes might be involved in this process. The formation of cell wall appositions in the paramural space might be a result of both secretory processes (i) and (ii) but is not a marker for PEN1 activity in mediating pre-invasion resistance. CU, cutin; CW, cell wall; PM, plasma membrane; PS, paramural space; AP, appressorium; S, spore; PP, penetration peg, P, phosphorylation.

For this reason, SYP122 in addition to PEN1 might be involved in the delivery of cell wall material to the cell periphery (Assaad et al. 2004; see Figure 26).

Despite the potential functional overlap of PEN1 and SYP122, I have shown that PEN1 but not SYP122 is active in pre-invasion resistance against *B. g. hordei* (Figure 19).

Because both syntaxins PEN1 and SYP122, as well as several tested non-functional variants of PEN1, were able to accumulate in cell wall appositions beneath attempted *B. g. hordei* entry sites (Assaad et al. 2004, and data not shown), focal accumulation is not a marker for PEN1 activity in pre-invasion resistance. Pre-invasion resistance and the focal accumulation at callose-containing cell wall appositions might even represent two independent processes. It is conceivable that a shared function of PEN1 and SYP122 directs callose precursors- and/or callose synthase-containing vesicles to the site of attempted *B. g. hordei* ingress to constitute the formation of papillae. This mechanism may involve exosomal-like secretion processes to translocate membranous, vesicle-like structures across the plasma-membrane to the paramural space. Since *syp122* single mutants display a primary cell wall defect and *pen1/syp122* double mutants are severely dwarfed and necrotic in the absence of pathogens, both PEN1 and SYP122 might have an additional general function in secretion of cell-wall material in unchallenged tissue (Assaad et al. 2004).

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Appendix A. List of Figures

Appendix B. List of Tables

Appendix C. Danksagungen

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Appendix D. Lebenslauf

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Appendix E Erklärung

„Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Paul Schulze-Lefert betreut worden."

Köln, im Mai 2007