

## Article Addendum

# Ready to fire

## Secretion in plant immunity

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Effective recognition of pathogens and rapid execution of immune responses are essential for the survival of living organisms. Cell-autonomous immune responses of animal and plant cells rely on pattern recognition receptors that can distinguish self from non-self structures and that are able to activate a molecular execution machinery that ultimately terminates most pathogen attacks. Reminiscent of the situation in mammalian T cells, accumulating evidence points to a key role of vesicle trafficking and exocytosis in plant innate immunity. In this context, our recent finding that ternary soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complexes comprising PEN1, SNAP33 and VAMP721/722 function at pathogen entry sites is instrumental in understanding the execution of plant immune responses at the cell periphery. Our study further revealed unexpected overlapping functions of the same SNARE complexes in disease resistance and development. Here, we discuss the potential identity of cargo delivered through the PEN1-SNAP33-VAMP721/722-dependent secretory pathway and the necessity for a tight regulation of SNARE complex formation to avoid unintentional release of toxic load.

Innate immunity of plants to obligate biotrophic powdery mildew fungi can be conceptually divided into two spatially and temporarily distinct layers that are under separate genetic control.<sup>1</sup> Pre-invasive defences combat penetration attempts of the pathogen at the periphery of attacked host epidermal cells. This defence mechanism often prevents successful colonization of the first host cell and leads to early termination of fungal pathogenesis. In contrast, post-invasive defences restrict further proliferation of the parasite once the first host cell has been colonized.<sup>2</sup> Mutational analysis in *Arabidopsis* revealed that pre-invasive defences against powdery mildews rely on

a set of *PENETRATION* (*PEN*) genes while post-invasive defences depend on components of a pathway governed by the signaling molecule salicylic acid (SA) as well as three sequence-related regulatory proteins (EDS1, PAD4, SAG101) that act in homo- and/or heteromeric complexes.<sup>2-4</sup> Three *PEN* genes have been cloned to date: *PEN2* encodes a peroxisome-associated glycosyl hydrolase-like protein and *PEN3* a plasma membrane-resident ABC transporter.<sup>2,4</sup> Both polypeptides are thought to co-function in a pathway that is distinct from *PEN1*-mediated resistance responses (see below). A recently discovered thioglycoside-cleaving activity of *PEN2* suggests that the *PEN2* reaction product is derived from indole glucosinolates, and this compound might be translocated from the cytosol to the extracellular space by *PEN3* activity (Bednarek and Schulze-Lefert, unpublished).

*PEN1* encodes a plasma membrane-resident syntaxin (*SYP121*) that is a member of the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) superfamily.<sup>3</sup> In eukaryotes, this protein family is known to mediate membrane fusion events, suggesting that vesicle trafficking and possibly exocytosis, contribute to antifungal pre-invasion defences. Recent findings indicate that virulent bacteria manipulate the plant secretory pathway by degrading an adenosine diphosphate ribosylation factor guanine nucleotide exchange factor (ARF-GEF) to suppress immune responses,<sup>5</sup> thereby demonstrating that regulatory components of the plant secretory pathway can become a direct target during microbial pathogenesis. We have now shown by biochemical analysis that *PEN1* indeed forms SDS-resistant ternary SNARE complexes with two further types of SNARE proteins, synaptosomal-associated protein of 33 kDa (SNAP33) and vesicle-associated membrane protein (VAMP) 721 or VAMP722.<sup>6</sup> These results corroborate the idea that *PEN1* function in immunity involves the targeted fusion of plant endomembrane compartments with the plasma membrane. Intriguingly, *PEN1*, SNAP33 and VAMP721/722 become all concentrated at fungal attack sites,<sup>6</sup> implying a focal delivery and discharge of cargos at infection sites to restrict pathogen entry by locally reinforcing the plant cell wall and/or directly secreting toxic compounds (Fig. 1). This is conceptually and mechanistically reminiscent of the execution machinery of activated T cells in vertebrates involving directional secretion of cytokines in helper T cells or focal secretion of cytolytic molecules in cytotoxic T cells to the contact sites with target cells.<sup>7,8</sup>

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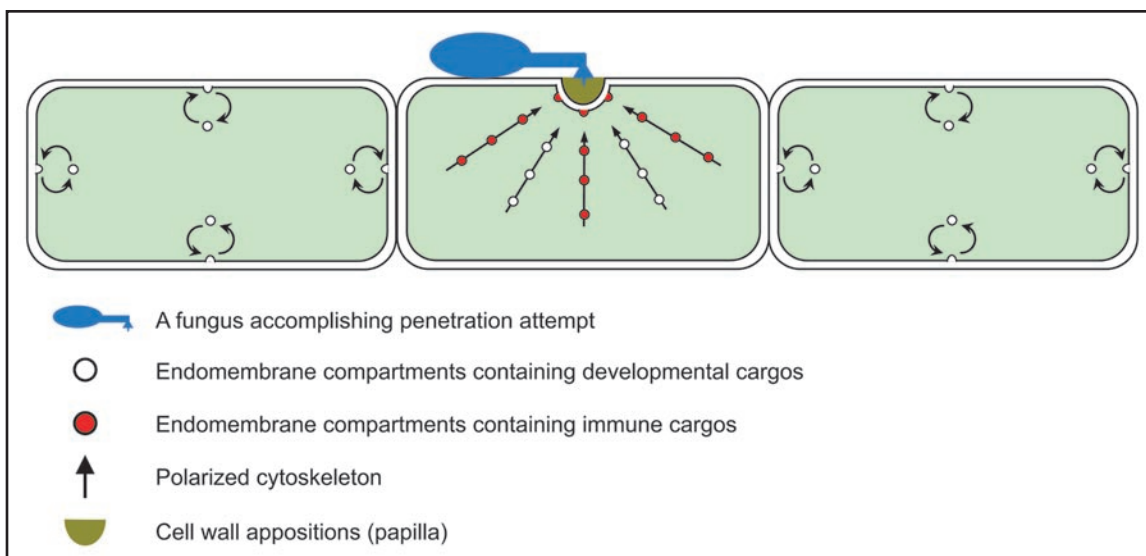


Figure 1. Cell-autonomous immunity in plants. In response to pathogens, plant cells under attack express specific immune responses to transfer and release developmental cargoes for cell wall expansion or remodeling and/or immune cargoes to directly restrict pathogen growth. The specific immune interactions between plant cells and pathogens are supposedly driven by cytoskeletal polarization and transcriptional reprogramming after the detection of non-self molecules expressed by pathogens.

What could be the cargo delivered to pathogen attack sites and locally concentrated by formation of the PEN1-SNAP33-VAMP721/722 ternary SNARE complex at the plasma membrane? It has long been noticed that plant cells secrete a diverse range of (poly-) peptides and small molecules to directly kill parasites or restrict pathogen growth. Pathogenesis-related (PR) proteins comprise members of 17 distinct polypeptide families exhibiting broad structural and functional diversity.<sup>9</sup> Acidic forms of PR proteins are typically secreted in response to pathogen attack whilst basic PR proteins accumulate in the plant cell vacuole.<sup>9</sup> Members of some PR protein families directly enhance host defenses through anti-microbial activity, but the exact action mechanism(s) of most PR proteins in immunity remains elusive to date.<sup>9</sup> In addition to secreted polypeptides, preformed (phytoanticipins) and de novo synthesized toxic plant secondary metabolites (phytoalexins) can also limit microbial growth.<sup>10,11</sup> The compromised disease resistance in *pen1* and *VAMP721*<sup>+/-</sup> *VAMP722*<sup>-/-</sup> as well as *VAMP721*<sup>-/-</sup> *VAMP722*<sup>+/-</sup> mutant plants strongly suggest that the freight delivered by VAMP721/722 endomembrane compartments encompasses molecules with direct or indirect anti-microbial activities.<sup>6</sup> Thus, PR proteins as well as toxic low molecular weight compounds can be considered as candidate load. Our analysis revealed that the endomembrane compartments containing VAMP722 congregate around attempted pathogen entry sites at approximately 12–15 h after inoculation. Leaf transcripts of several barley PR genes (*PR-2*, *-3*, *-4* and *-5* but not *PR-1*) were found to markedly increase in abundance as early as 6 h post inoculation with *Blumeria graminis* f. sp. *hordei* conidiospores.<sup>12</sup> If one assumes a similar rapid onset of PR gene transcription in Arabidopsis in response to *B. graminis* f. sp. *hordei* conidiospore challenge, congregated VAMP722 endomembrane compartments in single leaf epidermal cells beneath incipient fungal entry sites could indeed contain PR protein cargo. Of note, transcripts of several PR genes were preferentially or exclusively activated in leaf epidermal or mesophyll tissue in response to powdery mildew attack,<sup>12</sup> predicting that VAMP722 endomembrane compartments transport besides shared also unique pathogen-induced

cargos in these two leaf cell types. Recently, it has been shown that tobacco syntaxin SYP132 is required for the secretion of PR-1 during anti-bacterial resistance responses.<sup>13</sup> This indicates that at least a subset of PR proteins is exported by a distinct secretory pathway engaging SYP132 rather than the PEN1 (SYP121) syntaxin. How many of the secretory PR proteins are translocated into the apoplast by different SNARE complexes (via different syntaxins and/or VAMPs) remains to be tested.

Delayed focal deposition of the polyglucan callose beneath incipient fungal entry sites in the space between the plant cell wall and the plant plasma membrane in *VAMP721/722* co-silenced plants<sup>6</sup> indicates that cell wall-reinforcing molecules likely comprise yet another cargo class, namely cell wall precursors and/or enzymes for cell wall biosynthesis. Clearly, a complete inventory of VAMP721/722 endomembrane cargo will require the enrichment and analysis of the intracellular compartments marked by these SNARE proteins. The molecular composition of rat neuronal vesicles has only recently been reported, highlighting that the examination of vesicular compartments is a major experimental endeavor.<sup>14</sup> In Arabidopsis, distinct root cell types can be marked *in planta* through the use of cell-type specific promoters that drive the expression of the fluorochrome GFP in transgenic plants. Preparation of root protoplasts from such lines and subsequent flow cytometry permitted the rapid and efficient purification of single root cell types.<sup>15</sup> Similarly, flow cytometry enabled to further purify fluorescently labeled vesicles from a mixed vesicle population.<sup>16</sup> Therefore, using transgenic plants expressing GFP-VAMP722 driven by native regulatory sequences, GFP-VAMP722 compartments might be enriched by combining differential gradient centrifugation and flow cytometry. This enrichment of VAMP722 compartments possibly allows the identification of cargo proteins by mass spectrometry. In contrast to prokaryotes, eukaryotic cells restrict distinct cellular functions in different organelles by compartmentalizing enzymes and substrates: for example, DNA duplication occurs in the nucleus, respiration in the mitochondrion and photosynthesis in the chloroplast. Therefore, the identification of the

origin of endomembrane compartments containing GFP-VAMP722 could also indirectly hint at potential cargos transported by the PEN1-SNAP33-VAMP721/722 secretory pathway.

In many cases, PR proteins and toxic antimicrobial secondary metabolites have per se a broad activity spectrum against different parasites.<sup>9-11</sup> Although the *vamp721 vamp722* double mutant is lethal, *VAMP721<sup>+/-</sup> VAMP722<sup>-/-</sup>* plants are super-susceptible to the virulent *Hyaloperonospora parasitica* oomycete and *VAMP721<sup>-/-</sup> VAMP722<sup>+/-</sup>* plants show enhanced entry of *Erysiphe pisi*, a powdery mildew fungus that in nature colonizes pea plants.<sup>6</sup> This may suggest that VAMP721- and VAMP722-endomembrane compartments transport different cargo molecules. However, super-susceptibility of both *VAMP721<sup>+/-</sup> VAMP722<sup>-/-</sup>* and *VAMP721<sup>-/-</sup> VAMP722<sup>+/-</sup>* genotypes to the adapted powdery mildew fungus *Golovinomyces orontii*,<sup>6</sup> which has effectively overcome pre-invasive resistance, implies that immune cargos delivered by both VAMPs are largely overlapping during post-invasive resistance responses. How can these seemingly conflicting data be rationalized? *H. parasitica* colonizes mainly leaf mesophyll cells whilst powdery mildews infect only epidermal cells. Thus, the differential super-susceptibility phenotype of *H. parasitica* and *E. pisi* on *VAMP721<sup>+/-</sup> VAMP722<sup>-/-</sup>* or *VAMP721<sup>-/-</sup> VAMP722<sup>+/-</sup>* genotypes could reflect a differential relative abundance of VAMP721 and VAMP722 compartments in epidermal and mesophyll cells rather than differential vesicle cargo. Alternatively, the differential distribution of different PR proteins in epidermal or mesophyll cells<sup>12</sup> could also determine the differential activity of VAMP721 or VAMP722 in response to distinct pathogens. Relief from post-invasive *G. orontii* growth restriction in both *VAMP721<sup>+/-</sup> VAMP722<sup>-/-</sup>* and *VAMP721<sup>-/-</sup> VAMP722<sup>+/-</sup>* genotypes could reflect a concerted direct and indirect (systemic) defense action of epidermal and mesophyll tissue at later time points during fungal colonization.

Focal congregation of randomly scattered vesicle-like structures containing VAMP722 in response to pathogen attack (Fig. 1) hints to an involvement of a polarized cytoskeleton in the immune activity of the PEN1-SNAP33-VAMP721/722 secretory pathway. Indeed, pharmacological and genetic disruption of actin polymerization leads to compromised disease resistance.<sup>17,18</sup> Since the toxic molecules that should be safely transferred in vesicle-like containers are presumably also harmful to host cells, it might be essential to tightly and finely regulate the release of cargos in host cells. Several important fusion regulators such as Rab27a and Munc13-4 have been found to be essential for the killing activity of mammalian cytotoxic T cells.<sup>19</sup> In addition, regulated exocytosis in mammalian neuronal cells is known to be modulated by or even dependent on changes in calcium levels.<sup>20</sup> Since pathogen-associated molecular patterns (PAMPs) or elicitors such as Pep-13, cryptogin and oligogalaturonides rapidly modulate the cytosolic calcium levels in plant cells,<sup>21-23</sup> the potential role of calcium in fine tuning immune secretion in plant cells deserves future examination. Arabidopsis AtSYP122 and tobacco NtSyp121 were reported to be differentially phosphorylated in response to external elicitors such as flg22, a peptide surrogate of bacterial flagellin, and Avr9, a cysteine-rich fungal effector,<sup>24,25</sup> suggestive of a phosphorylation-dependent regulation of plant secretion events. Using transgenic Arabidopsis lines expressing PEN1 variants that prevent or mimic phosphorylation of N-terminal serine residues, we have recently found experimental support for the hypothesis that phosphorylation at the N-terminal residues is critical for full PEN1 activity in disease resistance to *B. graminis* f.sp. *hordei* (Pajonk S, Schulze-Lefert

P, unpublished).

Notably, our recent study revealed a second function of VAMP721/722 in plant growth, indicated by the lethality of homozygous *vamp721 vamp722* double mutants and severe dwarfism upon *VAMP721/VAMP722* co-silencing.<sup>6</sup> Likewise, double mutants in *PEN1* and its closest relative, *SYP122*, are seriously retarded in growth, and homozygous *SNAP33* mutants are embryo lethal.<sup>26,27</sup> This and the delayed deposition of cell wall appositions in *pen1* and *VAMP721/722*-silencing plants<sup>6,26</sup> strongly suggest cell wall precursors and/or cell wall-synthesizing enzymes as potential constitutive cargos delivered via VAMP721/722 endosomal compartments for cell expansion and cell wall remodeling (Fig. 1), and possibly also during immune responses. However, an inducible change in freight upon pathogen challenge can not be ruled out at present. It has been shown that the SA-dependent defense is activated by the lack of pathogen-inducible callose deposition at papilla.<sup>28,29</sup> Therefore, the compromised immunity in *VAMP721<sup>+/-</sup> VAMP722<sup>-/-</sup>* or *VAMP721<sup>-/-</sup> VAMP722<sup>+/-</sup>* plants<sup>6</sup> implies the delivery of SA-independent immune cargos by VAMP721/722 endomembrane compartments in response to pathogen attack. In this context, it will be of interest to understand how an infected host cell furnishes its cellular architecture for focal secretion and, simultaneously, controls the loading of potentially distinct cargos to the same cellular destination compartments containing VAMP721/722.

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