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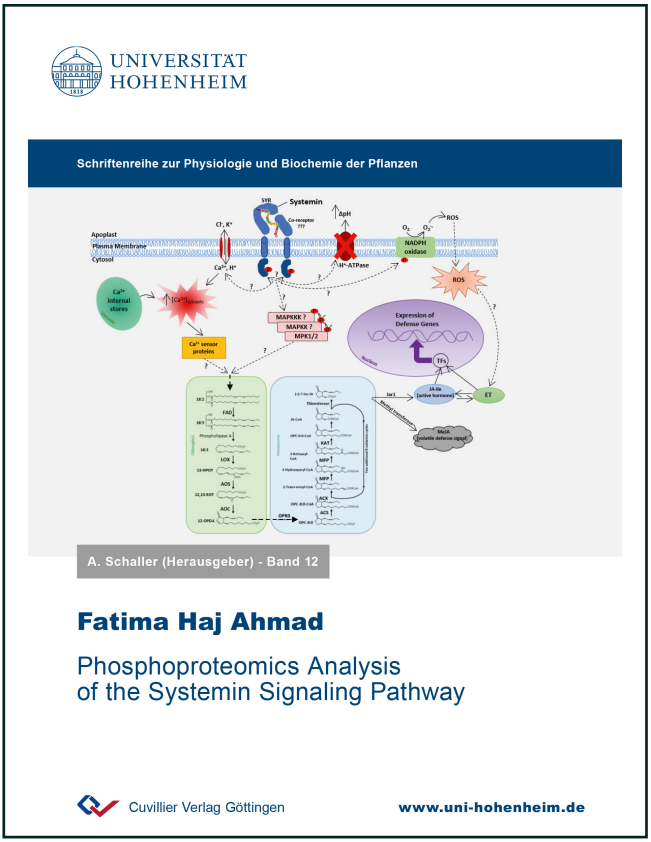
Phosphoproteomics Analysis of the Systemin Signaling Pathway



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Summary

One of the key players involved in herbivore and wound defense responses in tomato is Systemin. It was the first signaling peptide identified in plants in 1991, but the proteins and mechanisms involved in Systemin perception and signal transduction are still poorly understood. To address Systemin-induced signaling events, a phosphoproteomic profiling study involving time-course stimulation of *Solanum peruvianum* cell suspension cultures with Systemin and its inactive analog A17 was performed to reconstruct a Systemin-specific kinase/phosphatase signaling network. The time course analysis of Systemin-induced phosphorylation patterns revealed early events at the plasma membrane, such as dephosphorylation of H^+-ATPase, rapid phosphorylation of NADPH-oxidase and Ca^{2+}-ATPase. Later responses involved transient phosphorylation of small GTPases and vesicle trafficking proteins, as well as transcription factors. Based on a correlation analysis of Systemin-specific phosphorylation profiles, substrate candidates for 56 Systemin-specific kinases and 17 phosphatases were predicted including several receptor kinases as well as kinases with downstream signaling functions, such as MAP-kinases. A regulatory circuit for plasma membrane H^+-ATPase was predicted and confirmed by *in vitro* activity assays. In this regulatory model it is proposed that upon Systemin treatment, H^+-ATPase LHA1 is rapidly de-phosphorylated at its C-terminal regulatory residue T955 by phosphatase PLL5, resulting in the alkalization of the growth medium within two minutes of Systemin treatment. Further, it is suggested that the H^+-ATPase LHA1 is re-activated by MAP-Kinase MPK2 later in the Systemin response. MPK2 was identified with increased phosphorylation at its activating TEY-motif at 15 minutes of treatment and the predicted interaction with LHA1 was confirmed by *in vitro* kinase assays.

The Systemin signaling pathway was addressed also by studying the function of Systemin-induced receptor-like kinases (RLKs), which were selected from the phosphoproteomics data set. The relevance of these candidates as well as of the known

SYR1, SYR2 and PORK1 receptors for early and late Systemin-induced signaling events was analyzed *in vivo* in tomato plants and in *S. peruvianum* cell suspension cultures by creating loss-of-function mutants. An essential function for SYR1 for Systemin perception and early Systemin responses (alkalization of the growth medium) was confirmed in cell cultures. PORK1 function was found to contribute to early Systemin signaling events as well, in addition to its described role in the induction of late responses, including the induction of the proteinase inhibitor II wound response marker. Finally, it is proposed that Systemin crosstalk with other phytohormones, namely ABA and PSK, is mediated through LRK10L1.2 and PSKR2 receptors, respectively.

Zusammenfassung

Systemin ist einer der Schlüsselakteure der Wundantwort bei Tomaten. Es war das erste in Pflanzen identifizierte Peptid-Signal im Jahr 1991. Dennoch sind die Proteine und Mechanismen, die an der Systemin-Wahrnehmung und Signaltransduktion beteiligt sind, bislang kaum verstanden. Zur Untersuchung der Systemin-induzierten Signalwege wurde eine Phosphoproteomik-Profilierungsstudie durchgeführt, die eine zeitliche Stimulierung einer Suspensionskultur von *S. peruvianum* mit Systemin und dessen inaktivem Analogon A17 beinhaltete, was die Rekonstruktion eines Systemin-spezifischen Kinase/Phosphatase-Signalisierungsnetzwerkes erlaubte. Die Zeitverlaufsanalyse Systemin-induzierter Phosphorylierungsmuster zeigte frühe Ereignisse an der Plasmamembran, wie die Dephosphorylierung der H^+-ATPase und eine schnelle Phosphorylierung der NADPH-Oxidase und Ca^{2+}-ATPase. Spätere Antworten umfassten die transiente Phosphorylierung von kleinen GTPasen und Vesikeltransportproteinen sowie Transkriptionsfaktoren. Basierend auf einer Korrelationsanalyse Systemin-spezifischer Phosphorylierungsprofile konnten mögliche Substrate für 56 Systemin-spezifische Kinasen und 17 Phosphatasen vorhergesagt werden, darunter mehrere Rezeptor-Kinasen sowie Kinasen mit nachgeschalteten Signalfunktionen wie MAP-Kinasen. Ein Regelkreis für die Kontrolle der H^+-ATPase der Plasmamembran wurde vorhergesagt und durch in-vitro-Aktivitätstests bestätigt. In diesem Regulierungsmodell wird vorgeschlagen, dass es nach der Systemin-Behandlung zu einer schnellen Dephosphorylierung der H^+-ATPase LHA1 an ihrem C-terminalen regulatorischen Rest T955 durch die Phosphatase PLL5 kommt, was innerhalb von zwei Minuten nach der Systemin-Behandlung zur Alkalisierung des Wachstumsmediums führt. Weiterhin wird vorgeschlagen, dass die H^+-ATPase LHA1 später in der Systemin-Reaktion durch MAP-Kinase MPK2 wieder aktiviert wird. MPK2 wurde 15 Minuten nach Behandlung mit erhöhter Phosphorylierung an seinem

aktivierenden TEY-Motiv identifiziert und die Interaktion mit LHA1 wurde mittels in-vitro Kinasetests bestätigt.

Der Systemin-Signalweg wurde weiterhin im Hinblick auf eine Beteiligung Systemin-induzierter, Rezeptor-artigen Kinasen (RLKs) untersucht, die aus dem Phosphoproteomik-Datensatz hervorgegangen waren. Die Bedeutung dieser Rezeptor-Kandidaten sowie der SYR1, SYR2 und PORK1 Rezeptoren für frühe und späte Systemin-induzierte Signalereignisse wurde *in vivo* in Tomatenpflanzen und *S. peruvianum*-Zellsuspensionskulturen mit Hilfe eines Funktionsverlust-Ansatzes getestet. Die Notwendigkeit von SYR1 für die Systemin-Wahrnehmung und frühe Systemin-Antworten (Alkalisierung des Wachstumsmediums) wurde bestätigt. Es konnte auch eine Beteiligung von PORK1 an frühen Systemin-induzierten Signalereignissen nachgewiesen werden, neben der bekannten Bedeutung von PORK1 für die Regulation der später Anworten, derunter die Induktion des Proteinaseinhibitor II Wundreaktion-Markers. Darüber hinaus wird in dieser Arbeit vorgeschlagen, dass der Systemin-Crosstalk mit anderen Phytohormonen, nämlich ABA und PSK, durch die Rezeptoren LRK10L1.2- bzw. PSKR2-Rezeptoren vermittelt wird.

1. Introduction

Plants form the basis of most food chains on the planet. They influence their environment by shaping weather patterns, providing flood protection, purifying water, and providing food. On the other hand, plant survival is influenced by the environment. In order to survive their ever-changing surroundings while being anchored to the ground, plants have evolved a myriad of strategies to deal with many environmental challenges including thermal stress, wounding, oxidative stress, pathogens and herbivore attacks (Rampitsch and Bykova 2012).

The most prevalent herbivores are herbivorous insects. Their interaction with plants dates back several hundred million years, which has given rise to the co-evolutionary hypothesis (Fürstenberg-Hägg et al. 2013). This hypothesis proposes that insect feeding on plants has been a determining factor in increasing species diversity in both herbivores and hosts (Ehrlich and Raven 1964). Insect herbivores have traditionally been divided into specialists (monophagous and oligophagous insects) that feed on one or more host species from the same family, or generalists (polyphagous insects), which feed on several hosts from different plant families (Fürstenberg-Hägg et al. 2013).

The strategies employed by plants to defend themselves against insect herbivores are very diverse. They include morphological and biochemical responses, which might be either constitutively produced or induced upon attack (Howe and Schaller 2008; Mello and Silva-Filho 2002; War et al. 2018).

1.1. Plant Defense Strategies

Plant morphological defense strategies represent the first defense line of physical barriers against pathogens and herbivores. For example, epicuticular wax films and crystals increase slipperiness preventing insects from populating leaf surfaces (Savatin et al. 2014). Some plants have thorns and spines, which act mainly against mammalian herbivores (Howe and Schaller 2008). Leaf toughness is a physical barrier that exerts a challenge for insects (Howe and Schaller 2008). Upon wounding it is reinforced by deposition and accumulation of macromolecules in the cell wall such as lignin, cellulose, suberin, and callose leading to an induced physical resistance in plants (Howe and

Schaller 2008; Pastor et al. 2018). Additionally, trichomes hinder small insects from contacting the leaf surface or limit their movement (Mitchell et al. 2016). It is reported that the density of trichomes increase on young developing leaves of some plant species under herbivore attack to protect the plant from possible future attacks by the second herbivore generation (Dalin and Björkman 2003). On the other hand, glandular trichomes can be considered as both morphological and chemical resistance factors. Glandular trichomes produce and store substances, such as Volatile Organic Compounds (VOCs) and secondary metabolites, which can repel insect herbivores or immobilize them on the leaf surface upon destruction (Howe and Jander 2008; Howe and Schaller 2008).

One of the biochemical and physical barriers are resin and latex, which are viscous organic materials stored under internal pressure in ducts or network of canals in plants (Levin 1976). When herbivore feeding destroys these structures, their contents is released to trap or poison attacking insects and seal the wound to protect it from invading pathogens (Levin 1976; LoPresti 2016).

The major chemicals involved in the biochemical defenses are secondary metabolites (Howe and Jander 2008). Beside their importance as medicinal drugs, poisons, flavors, and industrial materials, the primary function of these chemicals is in plant defense, where they act as deterrents for herbivorous insects, as toxins or as antinutritive substances that reduce the nutritional value of the plant material (Fürstenberg-Hägg et al. 2013; Taiz and Zeiger, 2010). They include a huge number of compounds, which are classified into phenolics, terpenoids or alkaloids (Howe and Schaller 2008; Taiz and Zeiger 2010).

Phenolics such as tannins, coumarins, and phenylpropanoids serve as defense compounds by repelling feeding herbivores, by protection against microbial infections, and as building blocks of lignin (Adeboye et al. 2014). Terpenoids are released from plants under herbivore attack and act as antifeedants, repellents, toxins or as modifiers of insect development (Fürstenberg-Hägg et al. 2013). They are major components of resin and VOCs (Taiz and Zeiger 2010; Yazaki et al. 2017). Volatile bursts of terpenoids can act directly as herbivore repellent, or indirectly by attracting predators that kill plant-feeding insects (Sabelis et al. 2007; Unsicker et al. 2009). VOCs emitted by herbivore-damaged plants into the atmosphere interact with undamaged parts of the same plant as well as the neighboring plants, alerting them to their current or future risk of damage by herbivores (Arimura and Pearse 2017). Alkaloids are believed to provide defense against insects, pathogens and mammalian herbivores because of their general toxicity,

deterrence capability and metabolic effects (Mithöfer and Boland 2012). Examples of alkaloids are caffeine, nicotine, morphine, strychnine, and cocaine (Mithöfer and Boland 2012).

Another kind of biochemical defense is the extrafloral nectar, which is secreted on plant leaves and shoots to attract predators and parasitoids serving as one of the indirect defense lines of plants (González-Teuber and Heil 2009). Extrafloral nectar secretion is constitutive in some plant species (Heil et al. 2004), and induced by wounding in others (Heil and Ton 2008). Central American *Acacia* species, for example, are obligately inhabited by symbiotic ants that nourish from constitutively secreted extrafloral nectar and serve as an army that defends their host from herbivore attacks (Heil et al. 2004).

Among the induced biochemical defense strategies in plants are inducible defense proteins that reduce the insect's ability to digest the plant causing amino acid deficiencies, which negatively affect their growth and development (Mithöfer and Boland 2012). The plants' defensive protein arsenal includes enzymes such as arginase and threonine deaminase isoforms (Gonzales-Vigil et al. 2011) that degrade dietary amino acids necessary for insect growth (Howe and Jander 2008; Zhu-Salzman et al. 2008). Enzyme inhibitors such as α-amylase and proteinase inhibitors (PIs) can hinder starch and protein digestion respectively by binding tightly to the digestive enzymes such as α-amylase, trypsin and chymotrypsin (Mithöfer and Boland 2012; Taiz and Zeiger 2010). Oxidative enzymes such as polyphenol oxidase (PPO) and lipoxygenase (LOX) covalently modify the dietary proteins through the production of reactive *o*-quinones and lipid peroxides, respectively (Howe and Jander 2008). Some proteins such as ascorbate oxidases are involved in the disturbance of the insects' gut redox state, which may cause proliferation of oxyradicals that damage proteins, lipids, and DNA of the insect (Howe and Jander 2008; Wang and Constabel 2004). Some of the defense proteins are toxic, they target and damage the peritrophic matrix protecting the midgut epithelium of the insect such as lectins, thionins, chitinases, cysteine proteases and leucine aminopeptidases (Bowles 1990; Zhu-Salzman et al. 2008).

1.2. Induced Innate Immunity against Herbivore Attacks

Induction of defense mechanisms in plants depends on the successful recognition of their enemies by a sophisticated innate immune system (Boller and Felix 2009). Danger signals are recognized by surface-localized Pattern Recognition Receptors (PRRs) that

activate resistance responses referred to as Pattern-Triggered Immunity (PTI; Gust et al. 2017). Danger signals include molecular patterns associated with microbes (bacteria, viruses, fungi or oomycetes), nematodes, parasites and herbivores that act as elicitors for plant defense. These elicitors are termed Microbe- or Pathogen-Associated Molecular Patterns (MAMPs/PAMPs; Gust et al. 2017), NAMPs (Manosalva et al. 2015), ParAMPs (Hegenauer et al. 2016), and HAMPs (Basu et al. 2018; Mithöfer and Boland 2008), respectively. A key feature of the molecular signatures of these elicitors is that they are not present in the host, and therefore characterized as 'non-self' (Gust et al. 2017). Endogenous elicitors produced as a result of the damage caused by invading organisms are described as Damage-Associated Molecular Patterns (DAMPs; Boller and Felix 2009).

PTI induced by different elicitors perception via their PRRs provides protection against invaders that are unable to subvert the immune system of a given plant (Andolfo and Ercolano 2015). Host-adapted plant predators such as generalist herbivores, however, have evolved effector proteins as weapons, which target this first defense line at different stages to suppress active immunity, thereby increasing host susceptibility and enabling them to colonize their host (Deslandes and Rivas 2012; Kushalappa et al. 2016). Plants, in consequence, have evolved a second line of resistance called Effector-Triggered Immunity (ETI), which is based on the highly specific direct or indirect interaction of pathogen effectors and the products of plant resistance R genes (Jones and Dangl 2006; Jones et al. 2016).

During feeding on their host plants, insects release a vast array of HAMP elicitors and effectors (Erb et al. 2012). These HAMPs and effectors may arise from insect oral secretions (OS; regurgitant), saliva, ventral eversible gland (VEG) secretions, digestive waste products (e.g., frass) or ovipositional fluids (Basu et al. 2018). They are diverse in structure including: (i) enzymes such as glucose oxidase, ATPase and ß-glucosidase (Eichenseer et al. 1999; Mattiacci et al. 1995), (ii) modified forms of lipids like Fatty acid–Amino acid Conjugates (FACs) such as volicitin (Alborn et al. 1997), (iii) sulfur-containing fatty acids such as caeliferins (Alborn et al. 2007) and (iv) peptides released from digested plant proteins such as inceptins that are proteolytic fragments of the chloroplastic ATP synthase γ-subunit (Schmelz et al. 2006). In addition, the endosymbiotic microbes living in guts of herbivorous insects can induce and manipulate plant defense responses with their released MAMPs and effectors (Acevedo et al. 2015; Chung et al. 2013). Very little is known about PRRs or R proteins that recognize HAMPs or herbivore effectors in plants, respectively (Acevedo et al. 2015; Basu et al. 2018).

Although a putative volicitin receptor in maize has been reported (Truitt et al. 2004), its identity is still unclear (Acevedo et al. 2015; Basu et al. 2018).

Most DAMPs are plant cell components that are released passively upon mechanical damage caused by herbivore-feeding, or by hydrolytic enzymes that are released upon herbivory, microbial or fungal infections, and therefore termed as 'altered self' (Gust et al. 2017). They include the oligomeric fragments of plant cell-wall pectin, termed oligogalacturonides (OGs), which are recognized by WALL-ASSOCIATED (RECEPTOR) KINASE1 (WAK1) in *Arabidopsis* (Ferrari et al. 2013; Kohorn 2016), cutin-derived ω-hydroxy fatty acid monomers (and their corresponding alcohols) and cellulose-derived cellobiose and likely higher-order cellodextrins (Boller and Felix 2009; Choi et al. 2014; Gust et al. 2017). In addition, intracellular molecules such as ATP and NAD^+, which are released into the apoplastic space upon cellular damage can be recognized as DAMPs (Gust et al. 2017). In *Arabidopsis*, the extracellular ATP (eATP) is sensed by the L-type lectin receptor kinase Does Not Respond To Nucleotides1 (DORN1) and is able to induce plant defense (Choi et al. 2014).

A group of plant peptides that are processed from their pro-proteins by proteolytic cleavage and secreted upon herbivore attack, wounding or microbial infection were considered earlier to be secondary endogenous DAMP elicitors (Gust et al. 2017; Yamaguchi and Huffaker, 2011). Recently, these peptides were referred to as phytocytokines, since their release is not necessarily linked to tissue damage (Gust et al. 2017). They include Systemin identified in tomato (Pearce et al. 1991), which is recognized by its leucine rich repeat receptor-like kinase (LRR-RLK) SYR1 (Wang et al. 2018) and hydroxyproline-rich systemins (HypSys) of several Solanaceous plants (Pearce 2011). Other examples include the Plant Elicitor Peptides (PEPs) originally identified in *Arabidopsis* and recognized by two closely related LRR-RLKs, *At*PEPR1 and *At*PEPR2 (Bartels and Boller 2015; Krol et al. 2010). The *Arabidopsis* PAMP-Induced Peptides (PIPs), which are recognized by the LRR-RLK RLK7 to trigger immune responses in a manner similar to *At*PEP1 and bacterial flagellin-derived peptide Flg22, are also considered among the group of these phytocytokines (Hou et al. 2014). It was found that both PEP and PIP cooperatively amplify the immune responses triggered by Flg22 in *Arabidopsis* (Hou et al. 2014), and PEP amplifies HAMP-induced defense responses in rice (Shinya et al. 2018). Figure 1.1 presents a diagram summarizing plant innate immunity induced against insect herbivore.

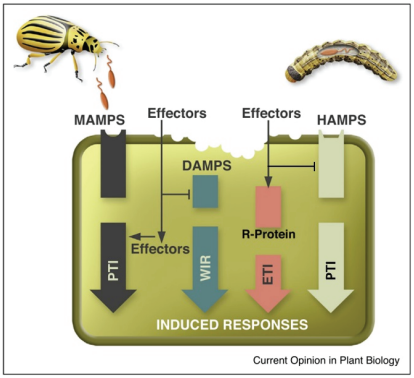

Figure 1.1: Model for Perception of Herbivore Elicitors.
HAMPs released from herbivorous insects as well as the MAMPs released from their microbial endosymbionts are recognized by their respective PRRs and trigger PTI. DAMPs also are recognized by cognate PRRs and induce wound-induced resistance (WIR). Herbivore and microbial effectors may suppress WIR and PTI. The microbial symbionts may be included in the oral secretions of some herbivore insects such as beetles (Chung et al. 2013) or may modify the expression of HAMPs or herbivore effectors, which can be recognized by plant R-proteins and trigger ETI (modified from Acevedo et al. 2015).

Increasing evidence suggests that the perception of elicitors from different sources such as HAMPs, MAMPs and DAMPs by their PRRs activate the same PTI responses via pathways that share the same intracellular signaling events but with differences in lag phases, amplitudes and kinetics (Choi and Klessig 2016; Ranf et al. 2011; Schmelz 2015). These intracellular signaling events include membrane depolarization, ion fluxes, intracellular Ca^{2+} influx, production of Reactive Oxygen Species (ROS), activation of the defense-associated Mitogen-Activated Protein Kinases (MAPKs), phytohormone biosynthesis and perception, activation of transcription factors, biosynthesis of defensive proteins and accumulation of defense-related metabolites (Choi and Klessig 2016; Schmelz 2015).

Recognition of effectors by plant R proteins induces ETI including intracellular signaling events that partially overlap with PTI with temporal and quantitative differences (Cui et al. 2015). ETI eventually induces the production of various antimicrobial proteins called Pathogenesis-Related (PR) proteins in and around the infected cell accompanied by the development of local Hypersensitive Response (HR; Fu and Dong 2013).