

Studies on recognition of pathogen-derived sphingolipids in plants

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CHAPTER 1

General introduction

Plant diseases caused by fungi, oomycetes, bacteria, viruses, and nematodes are a major constraint on global crop production and food security. Rising human populations, changing dietary preferences, and the expansion of agriculture into marginal environments are increasing the demand for stable yields, while climate change is simultaneously intensifying abiotic stress and reshaping the distribution and severity of biotic threats. Warmer temperatures and altered precipitation patterns can accelerate pathogen life cycles, promote the emergence of new pests, and expand the geographic range of existing pathogens into regions where crops and local varieties have not co-evolved with these threats (Skendžić et al., 2021). In South Korea, for example, shifts in spring temperature and rainfall are already advancing apple phenology and altering the regional suitability of production areas, illustrating how climate change is forcing farmers to adapt both crop species and management practices (Cho et al., 2021; Lee et al., 2023). Such environmental and socio-economic pressures highlight the importance of developing crops with durable, broad-spectrum disease resistance that can perform under variable and changing field conditions.

Plants rely primarily on an innate immune system rather than mobile immune cells and antibodies. A conceptual framework that has been highly influential is the “zigzag” model, which describes plant immunity as the integration of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). In this view, plant cells first detect conserved pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin or elongation factor Tu (EF-Tu), fungal chitin, or oomycete and fungal

cell wall components, through pattern-recognition receptors (PRRs) at the cell surface. This perception activates PTI, which restricts pathogen growth and often limits infection to minor, compatible or incompatible interactions. In turn, successful pathogens deliver effector proteins that suppress PTI, but these effectors can be recognized directly or indirectly by intracellular nucleotide-binding leucine-rich repeat (NLR) receptors, triggering a second layer of defense, ETI, that is often associated with strong transcriptional reprogramming and localized programmed cell death (Dodds and Rathjen, 2010; Cui et al., 2015). Recent work has emphasized that PTI and ETI are not independent but rather mutually potentiate each other, with cell-surface PRRs and intracellular NLRs engaging in extensive signaling crosstalk to generate robust resistance (Ngou et al., 2021; Yuan et al., 2023).

PTI is initiated when PRRs at the plasma membrane bind their cognate ligands. Most PRRs are receptor-like kinases (RLKs) or receptor-like proteins (RLPs) whose extracellular domains recognize PAMPs or endogenous damage-associated molecular patterns. Their intracellular kinase domains, or associated kinases, transduce the signal into the cytoplasm (Boller and Felix, 2009; Monaghan and Zipfel, 2012; Boutrot and Zipfel, 2017). The bacterial flagellin-derived peptide flg22 is perceived by FLAGELLIN-SENSING 2 (FLS2), whereas EF-Tu–derived elf18 is recognized by EF-Tu RECEPTOR (EFR); both receptors are leucine-rich repeat RLKs that form ligand-induced complexes with the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Chinchilla et al., 2007; Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Chitin, a major component of fungal cell walls, is recognized in Arabidopsis by the LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), which forms complexes with other LysM-containing partners (Miya et al., 2007; Zipfel, 2014). Perception of these ligands rapidly

initiates canonical early PTI responses, including ion fluxes, a burst of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) cascades, and large-scale transcriptional changes that ultimately enhance disease resistance (Bigeard et al., 2015; Gust et al., 2017).

Beyond classic protein- and carbohydrate-based PAMPs, plants also sense lipids and other hydrophobic molecules as danger signals. In animals, lipid-based microbe-associated molecular patterns such as lipopolysaccharide (LPS) are recognized by specific receptors and trigger innate immune responses (Raetz and Whitfield, 2002). In plants, knowledge of lipid-derived PAMPs has expanded more slowly, but several examples now demonstrate that lipid perception is an integral part of immune surveillance. The S-domain RLK LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) perceives medium-chain 3-hydroxy fatty acids derived from bacterial LPS, showing that plant PRRs can directly detect microbial lipids (Kutschera et al., 2019; Lenarčič et al., 2017). In addition, a variety of oxylipins, sphingolipids, and sterol derivatives function as signaling molecules during plant–microbe interactions, either as pathogen-derived virulence factors or as plant regulators of defense pathways (Berkey et al., 2012; Ali et al., 2018; Liu et al., 2021).

Sphingolipids are a structurally diverse class of lipids composed of a long-chain sphingoid base linked to a fatty acid via an amide bond and further modified at the C-1 position with polar head groups (Heung et al., 2006). In plants, the major complex sphingolipids are glycosylceramides and glycosyl inositol phosphorylceramides (GIPCs), which together account for a large fraction of plasma membrane lipids and are particularly enriched in detergent-insoluble membrane fractions often interpreted as nanodomains or “rafts” (Cacas et al., 2016; Cacas et al., 2013; Gronnier et al., 2016). Sphingolipids are

essential for viability and contribute to membrane organization, vesicle trafficking, and signal transduction (Michaelson et al., 2016; Liu et al., 2021). Their long-chain bases and ceramide backbones are also precursors of bioactive metabolites that can promote or suppress programmed cell death and modulate stress responses, including those induced during plant–pathogen interactions (Berkey et al., 2012; Ali et al., 2018).

In microbial pathogens, sphingolipids play equally important but distinct roles. Fungal sphingolipids, including glucosylceramides and inositol phosphorylceramides, are required for membrane integrity, morphogenesis, thermotolerance, and virulence, and they can modulate host immune responses (Heung et al., 2006; Wang et al., 2021; Bergia et al., 2025). A striking structural feature of many fungal glycosphingolipids is the presence of 9-methyl-branched sphingoid bases that are absent from plant sphingolipids. These 9-methyl-branched long-chain bases are found, for example, in cerebroside from diverse phytopathogens, where they serve as key epitopes recognized by host plants (Barreto-Bergter et al., 2011; Umemura et al., 2000). Early work demonstrated that cerebroside from the rice blast pathogen *Pyricularia oryzae*, and later from other fungi, induce phytoalexin accumulation and confer effective protection against rice blast disease when applied to plants or cell cultures (Koga et al., 1998; Umemura et al., 2000; Umemura et al., 2002). These findings established microbial glycosphingolipids as lipid-type elicitors and raised the possibility that plants may possess dedicated mechanisms to perceive pathogen-specific sphingolipid signatures.

More recently, systematic analyses of plant pathogenic fungi have highlighted that their sphingolipid metabolic pathways exhibit both conserved and unique features compared with those of plants and animals, with implications for host specificity and virulence (Zhu et al., 2023; Liu et al., 2021). For instance, changes in fungal sphingolipid

composition can alter cell wall architecture, appressorium formation, and the secretion of effector proteins, thereby influencing infection structures and disease outcomes (Wang et al., 2021; Bergia et al., 2025). From the host perspective, perturbations in plant sphingolipid metabolism, such as altered ceramide synthase activity or modified long-chain base unsaturation, can lead to spontaneous cell death and altered susceptibility to pathogens, underscoring the tight coupling between sphingolipid homeostasis and immune regulation (Markham et al., 2013; Ali et al., 2018; Gömann et al., 2021). Together, these studies position sphingolipids at the interface of pathogen virulence strategies and plant defense mechanisms.

The plant plasma membrane is not a homogeneous bilayer but is laterally compartmentalized into nanodomains enriched in particular lipids (including sphingolipids and sterols) and subsets of proteins. The raft concept was originally proposed in animal cells to describe dynamic microdomains formed by the clustering of sphingolipids and cholesterol, which serve as platforms for protein sorting and signal transduction (Simons and Ikonen, 1997). In plants, detergent-insoluble membrane fractions and advanced imaging approaches have revealed that sphingolipids, GIPCs, and specific proteins likewise segregate into microdomains (Cacas et al., 2016; Buré et al., 2014; Voxeur et al., 2014; Cassim et al., 2019). Super-resolution microscopy and live-cell imaging in *Arabidopsis* have shown that PRRs, transporters, and other signaling proteins reside in heterogeneous nanodomains whose size, stability, and composition are dynamically regulated by the cell wall, cytoskeleton, and environmental cues (Jarsch et al., 2014; McKenna et al., 2019; Jaillais and Ott, 2020). These nanodomains are thought to facilitate the assembly of receptor complexes and downstream signaling modules while also constraining or promoting receptor mobility depending on the context (Ott, 2017;

Cassim et al., 2019). Because sphingolipids are major constituents of these membrane regions, their composition and organization are likely to influence PRR function and thus the sensitivity and robustness of PTI.

An additional layer of regulation of cell-surface immune receptors arises from their biogenesis and quality control within the endomembrane system. Most PRRs are glycoproteins that enter the secretory pathway co-translationally, undergo N-linked glycosylation in the endoplasmic reticulum (ER), and are then further processed in the Golgi apparatus before being delivered to the plasma membrane (Strasser, 2016; Nagashima et al., 2018). N-glycosylation is catalyzed by the oligosaccharyltransferase (OST) complex, which transfers a pre-assembled oligosaccharide to specific asparagine residues within Asn-X-Ser/Thr motifs. The catalytic subunit of the OST, STT3, exists in two isoforms in many eukaryotes and can be part of different OST complexes with distinct substrate specificities (Kelleher and Gilmore, 2006). In *Arabidopsis*, the *STT3A*-containing OST complex is especially important for co-translational N-glycosylation and has been implicated in plant stress responses and development (Koiwa et al., 2003; Huttner and Strasser, 2012; Strasser, 2016).

Accumulating evidence indicates that proper N-glycosylation and ER quality control (ERQC) are crucial for the folding, stability, and function of PRRs. Studies of EFR showed that specific ERQC components—such as calreticulin 3, UDP-glucose:glycoprotein glucosyltransferase (UGGT), the SDF2–ERdj3B–BiP complex, and other chaperones—are required for its accumulation at the plasma membrane and for elf18 responsiveness (Li et al., 2009; Nekrasov et al., 2009). Mutations in genes encoding N-glycan processing enzymes and ERAD (ER-associated degradation) factors can similarly impair PRR abundance and immunity, revealing that misfolded or improperly

glycosylated receptors are efficiently removed to maintain proteostasis (Saijo, 2010; Hüttner and Strasser, 2012; Liu and Howell, 2016). More broadly, genetic and biochemical studies demonstrate that defects in N-glycosylation or in downstream modification steps affect plant growth, cell wall biosynthesis, abiotic stress tolerance, and pathogen resistance, highlighting the pleiotropic roles of protein glycosylation (Strasser, 2014; Strasser, 2016; Nagashima et al., 2018).

Recent structural and functional work has provided direct mechanistic links between specific N-glycans and immune receptor activation. For example, the cell-surface receptor kinase MIK2, which perceives SERINE-RICH ENDOGENOUS PEPTIDES (SCOOPs) and contributes to PTI, requires an N-glycan at Asn410 to form a stable ternary complex with its co-receptor BAK1 and the SCOOP12 peptide. Mutation of this glycosylation site impairs MIK2–BAK1 interaction and compromises SCOOP12-triggered immune responses (Jia et al., 2024). Such findings underscore that N-glycans are not merely passive structural decorations but can participate directly in receptor–coreceptor interfaces and thereby tune signaling output. They also suggest that the set of client proteins strongly dependent on specific OST subunits and ERQC components may include multiple PRRs and co-receptors, potentially linking N-glycosylation machinery to broad aspects of plant immunity.

In parallel, work on pathogen-derived sphingolipid perception has revealed a dedicated recognition system in Arabidopsis. The ceramide *Phytophthora infestans*–ceramide D (Pi-Cer D), purified from the oomycete *P. infestans*, elicits defense responses in Arabidopsis and enhances resistance to this pathogen (Kato et al., 2022; Monjil et al., 2024). Pi-Cer D is cleaved in the apoplast by the ceramidase NEUTRAL CERAMIDASE 2 (NCER2), releasing a 9-methyl-branched sphingoid base that is recognized by the lectin

receptor-like kinase RESISTANT TO DFPM-INHIBITION OF ABSCISIC ACID SIGNALING 2 (RDA2) at the plasma membrane (Kato et al., 2022). Loss-of-function mutations in NCER2 or RDA2 reduce responsiveness to Pi-Cer D and increase susceptibility to infection, indicating that plants can detect a microbe-specific sphingoid base as a non-self molecular pattern and translate this recognition into effective immunity (Kato et al., 2022; Zhu et al., 2023). These discoveries firmly establish that pathogen-derived sphingolipids, including ceramides and glycosphingolipids with 9-methyl-branched bases, function as bona fide PAMPs in plant–oomycete and plant–fungal interactions (Umemura et al., 2000; Monjil et al., 2024; Zhu et al., 2023).

Despite this progress, many aspects of sphingolipid-triggered immunity remain poorly understood. Key open questions include how widespread sphingolipid recognition is across plant species and pathogen taxa, how different sphingolipid structures are discriminated at the molecular level, and how lipid perception is integrated with other immune signaling pathways. Moreover, although it is now clear that N-glycosylation and ERQC are essential for the biogenesis and function of numerous PRRs, it is largely unknown how the protein glycosylation machinery contributes specifically to the perception of lipid-type PAMPs such as sphingolipids. Given that many PRRs and co-receptors are highly glycosylated and reside in sphingolipid-rich nanodomains at the plasma membrane, the OST complex and particularly its *STT3A* catalytic subunit are attractive candidates for regulating the abundance, folding, and activity of receptors involved in lipid perception.

Understanding how N-glycosylation via *STT3A* intersects with sphingolipid signaling could therefore provide fundamental insights into the molecular logic by which plants sense pathogen-derived lipids and could identify new strategies for engineering

disease-resistant crops. In the following chapter, I focus on the role of *Arabidopsis STT3A* in pathogen-derived sphingolipid recognition, using a combination of forward genetics, biochemical analysis, and molecular approaches to dissect how N-glycosylation contributes to the function of the NCER2–RDA2 pathway and broader PTI signaling.

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CHAPTER 2

***STT3A* is required for recognition of pathogen-derived sphingolipids in Arabidopsis**

INTRODUCTION

Plants have evolved highly sophisticated and multilayered immune systems that enable them to detect and respond to an enormous diversity of pathogens present in the environment (Dodds et al., 2024). These systems are essential for plant survival because, unlike animals, plants are sessile and cannot escape from invading microbes. The first step in plant immune responses is the recognition of pathogen-associated molecular patterns (PAMPs)—structurally conserved molecules derived from bacteria, fungi, oomycetes, and viruses (Nejat and Mantri, 2017; Segonzac and Zipfel, 2011). PAMP perception is mediated by pattern recognition receptors (PRRs), which are typically plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Activation of PRRs initiates pattern-triggered immunity (PTI), a central and evolutionarily ancient component of plant defense that restricts pathogen entry and suppresses colonization (Bigeard et al., 2015; Jones and Dangl, 2006).

PTI signaling begins when PRRs bind their respective ligands and subsequently recruit co-receptors to form an active receptor complex. Well-characterized examples include the association of FLS2 with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) after flagellin perception and the interaction of CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) with LysM-containing receptors upon chitin recognition (Chinchilla et al., 2009; Miya et al., 2007; Ngou et al., 2022). These receptor complexes activate

downstream signaling cascades including ion fluxes, production of reactive oxygen species, MAPK activation, transcriptional reprogramming, and reinforcement of the cell wall. Despite extensive progress in identifying PRRs and their ligands, many aspects of PTI remain unresolved, particularly regarding the perception of chemically diverse PAMPs and the downstream networks that shape ligand-specific immune responses.

One notable example of a complex glycolipid conjugate is the lipopolysaccharide (LPS) found on the surface of Gram-negative bacteria. LPS may induce non-specific stress in plants at elevated concentrations due to its amphiphilic detergent-like character (Ranf et al., 2016). Ranf et al. identified the first receptor component of LPS, the receptor-like kinase LORE (LipoOligosaccharide-specific Reduced Elicitation), which mediates sensitive perception of *Pseudomonas* and *Xanthomonas* LPS (Ranf et al., 2015).

Among the various types of PAMPs, sphingolipids represent an emerging and relatively underexplored class of immunogenic molecules. Sphingolipids consist of a long-chain sphingoid base linked via an amide bond to a fatty acid and decorated with various polar head groups, giving rise to substantial structural diversity (Heung et al., 2006) (Figure 1). Notably, certain fungi and oomycetes produce sphingolipids that contain a 9-methyl-branched sphingoid base. This moiety is absent from plant sphingolipids and thus serves as a non-self signature recognized by plants to activate immune responses (Koga et al., 1998). Cerebrosides—glycosphingolipids containing this 9-methyl-branched sphingoid base—were first identified as elicitor molecules from the rice blast pathogen *Pyricularia oryzae*, revealing that pathogen-derived sphingolipids can function as potent activators of immunity (Koga et al., 1998; Umemura et al., 2000).

Although sphingolipids have been extensively studied in mammals and pathogenic fungi for their roles in membrane integrity, virulence, and pathogenicity, significantly less

is known about their function and metabolism in plant–pathogen interactions. Pathogenic fungi and oomycetes rely heavily on sphingolipid-mediated processes for infection structure development, host colonization, and stress adaptation. However, compared with proteinaceous PAMPs or carbohydrate-based elicitors such as chitin, the molecular mechanisms underlying sphingolipid perception by plants remain relatively poorly characterized (Zhu et al., 2023). Understanding how plants detect these structurally unique lipids is therefore essential for constructing a more complete picture of innate immune recognition.

Sphingolipids represent a class of lipids, consisting of a long-chain sphingoid base backbone linked to a fatty acid through an amide bond at the 2-amino group and to a polar head group at the C-1 position via an ester bond, distinguishing it from glycerolipids, which have a glycerol backbone (Heung et al., 2006). Sphingolipids play a pivotal role in a multitude of biological processes across diverse organisms, including animals, plants, and fungi (Zhu et al., 2023). Sphingolipids constitute a class of membrane lipids that perform vital structural and signaling functions in a wide array of organisms, spanning from yeast to animals (Wang et al., 2021). In fungi, sphingolipids are reported from *Saccharomyces*, *Candida*, *Histoplasma*, *Cryptococcus*, *Aspergillus*, *Neurospora*, *Schizosaccharomyces*, *Fusicoccum*, *Shizophyllum*, *Amanita*, *Hansenula*, *Lactarius*, *Lentinus*, *Penicillium*, *Clitocybe*, *Paracoccidioides*, *Agaricus*, and *Sporothrix* (Olsen and Jantzen, 2001).

A previous research has indicated that the loss of structural diversity of complex sphingolipids may impact the cellular environment, including both plasma membranes and cell walls, which could potentially contribute to an increased sensitivity to various environmental stresses (Koga et al., 2022). Current state of the knowledge regarding

metabolism and function of the sphingolipid metabolic pathway of pathogenic fungi affecting plants is still unclear (Zhu et al., 2023). A recently characterized sphingolipid PAMP is *Phytophthora infestans* ceramide D (Pi-Cer D), a ceramide-derived lipid purified from the oomycete *Phytophthora infestans* that promotes resistance in several plant species (Monjil et al., 2024). In *Arabidopsis thaliana*, Pi-Cer D is first cleaved by the apoplastic ceramidase NEUTRAL CERAMIDASE 2 (NCER2), generating a 9-methyl-branched sphingoid base that acts as the active elicitor. This sphingoid base is recognized by the plasma-membrane-localized lectin receptor-like kinase RESISTANT TO DFPM-INHIBITION OF ABSCISIC ACID SIGNALING 2 (RDA2), thereby initiating a robust immune response (Kato et al., 2022). This two-step recognition mechanism—initial cleavage of the pathogen lipid followed by receptor-mediated perception—highlights a unique strategy for lipid-based immune activation that differs mechanistically from the recognition of peptide or carbohydrate PAMPs.

Despite these advances, the full molecular framework underlying sphingolipid perception remains incomplete. In particular, the factors that modulate receptor biogenesis, ligand sensitivity, signal transduction, and specificity remain largely unknown. Sphingolipid-triggered immunity likely requires additional components beyond NCER2 and RDA2, including potential co-receptors, adaptor proteins, downstream kinases, trafficking factors, and regulators of receptor folding or membrane localization. Because lipid-based PAMPs differ structurally and biophysically from protein or glycan elicitors, they may impose distinct requirements on receptor maturation, particularly for extracellular domains that must bind hydrophobic ligands in an aqueous environment. This raises the possibility that specific aspects of the endoplasmic reticulum (ER) quality control machinery or N-glycosylation pathways may be critical for assembling functional

sphingolipid receptors, yet such connections have not been thoroughly investigated.

To address these challenges, forward genetic approaches provide powerful tools to identify components involved in sphingolipid-triggered immune signaling. Lumi-Map is a luciferase-based mutant screening platform that enables real-time monitoring of defense gene activation in response to elicitors, followed by rapid causal gene identification through whole-genome sequencing (Kato et al., 2020). Using this system, mutants with altered luminescence responses can be efficiently detected, allowing the discovery of novel genes required for perception or signaling.

In this study, I employed Lumi-Map (Figure 2) to identify previously unknown components of sphingolipid recognition in *Arabidopsis thaliana*. Through this screening approach, I identified *STAUROSPORIN AND TEMPERATURE SENSITIVE 3-LIKE A* (*STT3A*)—the catalytic subunit of the oligosaccharyltransferase complex responsible for co-translational N-glycosylation—as an essential factor in Pi-Cer D–induced immune signaling. I further investigated the role of *STT3A* in sphingolipid-triggered responses and pathogen resistance, revealing a previously uncharacterized connection between N-glycosylation and lipid-based immune perception. These findings expand our understanding of plant innate immunity and highlight the importance of ER-mediated protein maturation in the recognition of pathogen-derived sphingolipids.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis* (*Arabidopsis thaliana*) lines used in this study were in the Col-0 background. Seeds of the *stt3a-2* (SALK_058814), *rda2-3* (SALK_143489C), and *ncer2* (SALK_050305) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). The *rda2-4*, *rda2-5*, *rda2-4/RDA2-HA* and *ncer2-2* mutants were described previously (Kato et al., 2022). All seeds were surface sterilized using a sodium hypochlorite solution and incubated at 4°C in the dark for 4 days prior to seed sowing. Seeds were plated on Murashige and Skoog (MS) (Murashige and Skoog, 1962) liquid medium containing 0.5% (w/v) sucrose, B5 vitamin solution, and 2 mM MES (pH 5.7) and incubated at 23°C in the light (24-h photoperiod, 57 $\mu\text{mol m}^{-2} \text{s}^{-1}$). For seed propagation, *Arabidopsis* plants were grown on MS agar medium (MS salt, 1.5% sucrose, B5 vitamin solution, 2 mM MES [pH 5.7], and 0.8% agar) for 10 to 14 days, transferred to soil, and growth under continuous light at 23°C (57 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Soil-grown *Arabidopsis* plants were grown under controlled conditions (23°C, 10-h photoperiod, 57 $\mu\text{mol m}^{-2} \text{s}^{-1}$)

Genotyping T-DNA insertion lines

Genomic DNA was extracted from seedlings in sucrose lysis buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 300 mM sucrose. PCR products were obtained with KOD One PCR Master Mix (TOYOBO) and separated by electrophoresis on 1% (w/v) agarose gels. Left primer (LP) and border primer (BP) were used as forward primers, and right primer (RP) was used as the reverse primer (Table 2) to identify lines

homozygous for the respective T-DNA insertion. The PCR conditions were 98°C for denaturation, 55°C for annealing, and 68°C for extension.

Elicitors and chemicals

Pi-Cer D was purified from *Phytophthora infestans* as described by Monjil et al. (2024). Other elicitors and chemicals were acquired or purchased from commercial sources: flg22, elf18, and nlp24 (Life Technologies, Tokyo, Japan); chitin (C9752, Sigma); (4E,8E)-9-methyl-4,8-sphingadienine (9Me-Spd, NS440901; Nagara Science); 3-hydroxy decanoic acid (3-OH-FA, 24613; Cayman). Cello-oligosaccharides (COS) were prepared via hydrolysis of cotton linters or microcrystalline cellulose (Avicel PH 101, Sigma-Aldrich, Burlington, MA, USA).

Measurement of bioluminescence responses

Surface-sterilized seeds were incubated at 4°C in the dark for 4 days and sown in the wells of 96-well microplates (LuminuncTM Plates White F96; Thermo Fisher Scientific) containing 150 µl liquid MS medium supplemented with 50 µM D-luciferin, potassium salt (Biosynth). Seed germination was conducted under continuous illumination (23°C, 57 µmol m⁻² s⁻¹). Following a 7-day incubation period, the seedlings were treated with elicitors (100-fold dilution), and the 96-well plates were sealed with a plate seal (100-THER-PLT, Excel Scientific) instead of a plastic cover. Bioluminescence from each well was measured automatically and immediately after elicitor addition using a bioluminescence monitoring system (model CL96-4; Churitsu Electric Corp.) with a robotic plate conveyor (model CI-08L; Churitsu Electric Corp.). Bioluminescence data were analyzed using the software provided with the instrument (SL00-01; Churitsu

Electric Corp.).

Generation of F₂ progeny and whole-genome sequencing

F₁ progeny were generated by crossing the p*WRKY33-LUC* reporter line (W33-1B, the parental line of the mutants) (Kato et al., 2022) with each of the mutants identified in the screen. All F₁ plants were subsequently self-pollinated to obtain F₂ seeds. The bioluminescence responses of the F₂ seedlings were tested using Pi-Cer D treatment as described above. F₂ seedlings with the mutant phenotype were selected and their genomic DNA extracted. The same quantity of genomic DNA from 30 F₂ seedlings with the mutant phenotype was combined to obtain a bulk (pooled) DNA sample for MutMap analysis. For whole-genome sequencing, genomic DNA samples were extracted from young (3-week-old) leaves using a DNeasy Plant Mini Kit (Qiagen). Sequencing libraries were prepared for the L-08, L-13, and L-51 mutant lines using an Illumina TruSeq DNA LT Sample Prep Kit (Illumina). All libraries were sequenced as paired-end 150-bp reads. The libraries were sequenced on a HiSeq High-Output system. The whole-genome sequencing data have been deposited in DDBJ BioProject under accession number PRJDB20249.

MutMap analysis

The p*WRKY33-LUC* reporter line reference sequence was constructed by replacing nucleotides in Col-0 with those of the p*WRKY33-LUC* reporter line W33-1B (Kato et al., 2022). Bulk segregant analysis, as implemented in MutMap (Abe et al., 2012), was performed using the MutMap pipeline (<https://github.com/YuSugihara/MutMap>) (Sugihara et al., 2022). The Arabidopsis Col-0 reference genome was downloaded from <ftp://ftp.ensemblgenomes.org/pub/release->

36/plants/fasta/Arabidopsis_thaliana/dna/Arabidopsis_thaliana.TAIR10.dna.toplevel.fa.

Whole-genome sequencing of the three mutants identified 773 ± 334 (mean $\pm$ s.d.; range 398–1,038) SNPs relative to the *pWRKY33-LUC* reporter parental line W33-1B.

Plasmid construction and plant transformation

To complement the *stt3a* mutants (*stt3a-3*, *stt3a-4*, *stt3a-5*), a 10.7-kb genomic fragment containing the *STT3A* genomic coding region and its native promoter was amplified and inserted into pBIB-KAN at the *SalI* and *SacI* restriction sites using an In-Fusion HD Cloning Kit (Takara). The primer sequences used for plasmid construction are listed in Table 2.

The plasmids were introduced into *Agrobacterium* (*Agrobacterium tumefaciens*) strain GV3101::pMP90 by electroporation and used to transform *Arabidopsis* plants via the floral dip method (Clough and Bent, 1998). T₁ seeds were collected from these T₀ plants, and T₁ seedlings were grown on MS medium containing kanamycin (50 µg/ml) and carbenicillin (150 µg/ml) to select transgene-positive T₁ plants. T₂ seeds were then collected from the positive T₁ plants and sown on MS medium containing kanamycin; T₂ lines showing a 3:1 segregation ratio were thought to contain a single T-DNA. T₃ plants homozygous for the transgene were obtained and used for subsequent analysis.

MAPK activation assay

Seedlings germinated from surface-sterilized seeds were grown in 96-well microplates in MS liquid medium for 7 days. Samples were obtained from sphingolipid-treated seedlings at 15 and 30 min of treatment (0.17 µM Pi-Cer D, 0.5 µM 9Me-Spd) using the same method used for screening. All samples were immediately frozen in liquid

nitrogen. Proteins were extracted from the samples in extraction buffer (50 mM HEPES, pH 7.4, 5 mM EDTA, 0.5 mM EGTA, 50 mM β -glycerophosphate, 10 mM Na_3VO_4 , 10 mM NaF, 2 mM DTT). MAPK activation was monitored by immunoblot analysis using an antibody that recognizes the dual phosphorylation of the activation loop of MAPK (pTEpY). Phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204, Cell Signaling Technology), and rabbit monoclonal antibodies were used to detect phosphorylated MAPK according to the manufacturer's protocol (#9101, Cell Signaling Technology). Immunoblotting detection solution (ECL Prime Western Blotting Detection Reagents, Amersham) was used for signal detection. The PVDF membrane was stained with Coomassie Brilliant Blue (PageBlue Protein Staining Solution, Thermo Fisher Scientific) to verify equal protein loading.

RNA extraction and RT-qPCR

Seven-day-old seedlings were treated with sphingolipids, and samples were collected at the specified time points before being frozen in liquid nitrogen. Total RNA was extracted from the samples using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Following RNA extraction, first-strand cDNA was synthesized using Takara PrimeScript RT Master Mix (Takara). Quantitative PCR was conducted using rTaq DNA polymerase (TAP201, TOYOBO) and EvaGreen Dye (31000, Biotium, Inc.) on a CFX Connect Real-Time System (BIO-RAD) with the primers listed in Table 2. Relative gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ method, with *ubiquitin-conjugating enzyme 21* (*UBC21*, At5g25760) (Czechowski et al., 2005) serving as a reference for normalization.

Pathogen inoculation assay

The inoculation assay was performed as previously reported (Singkaravanit-Ogawa et al., 2021) with some modifications. *Colletotrichum higginsianum* strain MAFF_305635 was cultured on potato dextrose agar (BD Biosciences, Franklin Lakes, NJ) at 24°C in the dark. For plant inoculation, four drops (5 µl per drop) of a spore suspension containing 1.5×10^5 conidiospores/ml were placed onto one leaf of a 5- to 6-week-old Arabidopsis plant without punching. The plants were covered with a transparent lid to maintain high humidity conditions and placed in a growth cabinet at 23°C under a 10-h light/14-h dark photoperiod until the day of observation (4 dpi). The inoculated leaves were excised and photographed after 4 days for analysis. The lesion size (area) was quantified using ImageJ (<https://imagej.net/ij/>). The statistical significance of differences between means was determined using Tukey's test. In the figures, different lowercase letters indicate significant differences ($p < 0.05$).

Production of the anti-NCER2 antibody

A peptide from the C-terminus of NCER2 (ADVPPKSTFRR) with an N-terminal cysteine was chemically synthesized (Scrum). The antigen peptide was conjugated to a keyhole limpet hemocyanin carrier via SS-linkage through the N-terminal cysteine, and polyclonal antisera were raised in rabbits (Scrum). Anti-NCER2 antibodies were purified from the antisera by affinity chromatography using antigen-immobilized beads (HiTrap NHS-activated HP; GE Healthcare).

Extraction of apoplast wash fluid and detection of NCER2

Apoplast wash fluid (AWF) was extracted from the samples as described by Gentzel

et al. (2019), with minor modifications. Extraction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl) was introduced into leaves via syringe infiltration. The leaves were blotted dry with a paper towel, wrapped around a 1-ml pipette tip using Parafilm, and placed in a 15-ml conical tube. The tube was subjected to centrifugation at $1,000 \times g$ for 3 min at room temperature using a swing rotor. AWF proteins were precipitated by acetone precipitation and resuspended in extraction buffer containing 1% SDS. The protein content of the AWF was quantified using the Bradford method, and a 10 μ g equivalent of AWF proteins was separated by electrophoresis on a 12% (w/v) SDS acrylamide gel. Immunoblot analysis was performed with anti-NCER2 antibodies (1:2,000 dilution) and HRP-conjugated secondary antibodies (1:2,000 dilution).

Generation of RDA2-HA transgenic lines and immunoblot analysis

To analyze the post-translational modification of RDA2, the coding region of *RDA2* was fused to a C-terminal HA epitope tag and cloned into a binary vector under the control of the native *RDA2* promoter (Kato et al., 2022). The resulting construct was introduced into WT and *stt3a-3* background plants via *A. tumefaciens* (strain GV3101::pMP90)–mediated floral dip transformation. Transgenic T₁ seedlings were selected on kanamycin-containing MS plates and transplanted on the soil. After growing on the soil, transgenic T₁ plants were used for HA immunoblotting. Total proteins were extracted from four-week-old T₁ transgenic plants expressing *RDA2-HA* using extraction buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.25% NP-40. Samples were centrifuged at $13,000 \times g$ for 10 min at 4 °C, and supernatants were collected. Equal amounts of protein (20 μ g) were separated by SDS-PAGE and transferred to PVDF membranes. RDA2-HA was detected using anti-HA monoclonal antibody (3F10, 1:2,000 dilution, Roche)

followed by HRP-conjugated secondary antibody and chemiluminescence detection.

Treatment of deglycosylation enzymes with NCER2 and RDA2

Endo H (New England Biolabs, #P0702), PNGase F (#P0704), or PNGase A (#P0707) were used according to the manufacturer's instructions with minor modifications.

For NCER2, AWF was collected from Arabidopsis plants. AWF was denatured in the supplied denaturing buffer and heated at 99 °C for 10 minutes. Enzymatic reactions were carried out at 37 °C for 1 hour. After deglycosylation enzymes treatment in AWF, AWF was concentrated by acetone precipitation. The resulting protein pellet was resuspended in extraction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl) containing 1% SDS. The protein content of the AWF was quantified using the Bradford method, and equal amount of proteins were separated by SDS-PAGE and immunoblotted using anti-NCER2 antibodies.

For RDA2, total protein was extracted from Arabidopsis plants expressing RDA2-HA. Protein samples were denatured in denaturing buffer at 99 °C for 10 minutes, followed by treatment with PNGase A. After 1 hour of enzymatic digestion at 37 °C, the samples were resolved by SDS-PAGE and probed with anti-HA antibodies to detect RDA2-HA.

Accession numbers

The accession numbers of the Arabidopsis genes mentioned in this article are as follows: *STT3A* (At5g19690), *RDA2* (At1g11330), *NCER2* (At2g38010), and *WRKY33* (At2g38470).

RESULTS

Mutants showing low responses to Pi-Cer D were identified.

The expression of the Arabidopsis defense gene *WRKY33* is induced by diverse PAMPs, including the peptide flagellin 22 (flg22) and the sphingolipid Pi-Cer D (Denoux et al., 2008; Kato et al., 2022; Zheng et al., 2006) (Figure 3). Therefore, I used an Arabidopsis reporter line harboring the firefly *luciferase* gene under the control of the *WRKY33* promoter (p*WRKY33-LUC*) (Kato et al., 2022). I mutagenized the p*WRKY33-LUC* reporter line (the wild type, WT) with ethyl methane sulfonate and screened 10,000 M₂ seedlings in a previous study (Kato et al., 2022). In the present study, I selected and analyzed previously uncharacterized mutants from the same EMS population that exhibited reduced bioluminescence in response to Pi-Cer D. To select mutants, I first confirmed the Pi-Cer D-induced *WRKY33* expression (Figures 4A and 4B), then examined their responses to other PAMPs, which allowed us to classify the mutants according to their recognition patterns (Figure 4C). Among the mutants, L-08, L-13, and L-51 showed low bioluminescence responses to Pi-Cer D treatment: L-08 and L-51 exhibited minimal responses to Pi-Cer D, whereas L-13 showed a response that was approximately half that observed in WT at its peak (Figure 4A). I performed reverse-transcription quantitative PCR (RT-qPCR) to evaluate *WRKY33* expression levels in WT and mutant seedlings at 0 and 1 h of treatment with Pi-Cer D. The relative transcript accumulation of *WRKY33* was lower in all three mutants compared to WT specifically following Pi-Cer D treatment (Figure 4B).

To classify the mutants showing low bioluminescence responses to Pi-Cer D treatment, I investigated the responses of these mutants to four additional PAMPs: the 9-

methyl-branched sphingoid base (4E,8E)-9-methyl-4,8-sphingadienine (9Me-Spd), flg22, elf18, and chitin (Figure 4C). (4E,8E,10E)-9-methyl-4,8,10-sphingatrienine (9Me-Spt), a sphingoid base and a degradation product of Pi-Cer D, is a natural compound that is difficult to obtain in significant quantities. For this reason, I used 9Me-Spd, a commercially available structural analog of 9Me-Spt for the experiments. Flg22, a peptide derived from bacterial flagellin, elicits defense responses and is recognized by the receptor FLS2 (Chinchilla et al., 2006). Elf18 is the N-terminal fragment of the bacterial elongation factor Tu recognized by the EF-TU RECEPTOR (EFR) (Zipfel et al., 2006). Chitin is a component of the fungal cell wall that is recognized by the receptor CERK1 (Miya et al., 2007). All three mutants, L-08, L-13, and L-51, exhibited diminished induction of bioluminescence in response to treatment with Pi-Cer D, 9Me-Spd or elf18 compared to WT, whereas their responses to flg22 and chitin were similar to those of WT (Figure 4C). Therefore, I focused on these three mutants for further analysis.

I also examined the responses of the three mutants to nlp24, 3-hydroxydecanoic acid (3-OH-FA), and cello-oligosaccharides (COS) (Figure 5). Nlp24 is a peptide derived from necrosis- and ethylene-inducing peptide 1-like proteins (Albert et al., 2015). 3-OH-FA is perceived by the lectin receptor kinase LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) (Kutschera et al., 2019). COS are small carbohydrate molecules derived from the partial hydrolysis of cellulose that can elicit a broad-spectrum immune response against several pathogens (Chen et al., 2021; Kongala and Kondreddy, 2023). The mutants showed lower induction of bioluminescence in response to treatment with 9Me-Spd or COS compared to WT, whereas they had normal responses to treatment with nlp24 or 3-OH-FA (Figure 5). The similar responses to

various elicitors exhibited by the three mutants suggest that the causative genes of the three mutants are likely involved in the same pathway related to plant responses against Pi-Cer D, 9Me-Spd, elf18, and COS.

Mutation of *STT3A* results in the low response to Pi-Cer D

To identify the causal mutations of L-08, L-13, and L-51, I crossed each mutant line to the *pWRKY33-LUC* reporter line (the parental line of the mutants) to produce F₁ progeny, which were self-pollinated to obtain F₂ seeds. I then tested the F₂ segregating population derived from each mutant using Lumi-Map (Kato et al., 2020) to select F₂ seedlings with the mutant phenotype of a reduced bioluminescence response to Pi-Cer D treatment. Following Pi-Cer D treatment, I chose 30 F₂ individuals showing low bioluminescence responses for genomic DNA extraction. For each F₂ population, I obtained equal amounts of leaf material from each individual showing a mutant phenotype, mixed the materials together, and extracted DNA from this mixture. I sequenced the DNA samples from each mutant line to detect single nucleotide polymorphisms (SNPs) relative to the *pWRKY33-LUC* reporter line, whose genome was also sequenced. When I plotted the SNP-index as a function of SNP position, I identified a single genomic region with SNP-index values close to 1 for all three mutants. Importantly, the three mutants displayed one large SNP-index peak at the identical position (6 Mb) on chromosome 5 (Figure 6).

Based on the *pWRKY33-LUC* reporter line reference genome, the region with the highest SNP-index value in the L-08, L-13, and L-51 mutants contains multiple candidate genes, with the gene *STT3A* harboring mutations in all three mutants (Table 1). *STT3A* encodes an oligosaccharyltransferase that plays a pivotal role in the glycosylation of

diverse proteins (Cheng et al., 2022). The three mutants harbored different types of mutations: splicing junctions in L-08 and L-13, and an amino acid substitution in L-51 (Figure 7A; Table 1). Genetic complementation of the three mutants with a wild-type genomic *STT3A* fragment led to the recovery of a normal bioluminescence response following Pi-Cer D treatment (Figure 7B). These results demonstrate that the poor induction of the *pWRKY33-LUC* reporter to Pi-Cer D treatment in the L-08 (*stt3a-3*), L-13 (*stt3a-4*), and L-51 (*stt3a-5*) mutants is caused by mutations in *STT3A*. Furthermore, I obtained the Arabidopsis line *stt3a-2*, with a T-DNA insertion in *STT3A* (Figure 7A; Figure 8). This mutant showed compromised induction of *WRKY33* expression after treatment with Pi-Cer D, as determined by RT-qPCR (Figure 7C). As a negative control, I used *rda2-3*, a line with T-DNA insertion in *RDA2* gene, which supported no *WRKY33* expression in response to Pi-Cer D treatment (Figure 7C). These results suggest that *STT3A* is involved in defense signaling mediated by Pi-Cer D in Arabidopsis. To examine the expression of *STT3A* in the mutant alleles, I performed RT-qPCR using primers targeting the exon region of *STT3A* (Figure 7A; Figure 9). RT-qPCR analysis revealed markedly reduced *STT3A* transcript levels in *stt3a-3* and *stt3a-4* compared to WT, whereas *stt3a-5* showed similar expression to WT. The T-DNA insertion line *stt3a-2* also showed diminished *STT3A* transcript levels compared to Col-0.

***STT3A* is required for defense signaling in Arabidopsis following sphingolipid recognition**

To determine the role of *STT3A* in defense signaling, I evaluated the expression of defense-related genes and the activation of Mitogen-Activated Protein Kinase (MAPK) signaling following Pi-Cer D treatment of WT, *stt3a-3* (L-08), *stt3a-4* (L-13), and *stt3a-*

5 (L-51) seedlings. *PENETRATION 2* (*PEN2*) encodes a glycosyl hydrolase that localizes to peroxisomes and acts as a component of an inducible preinvasion resistance mechanism (Lipka et al., 2005). *CYTOCHROME P450 FAMILY 81 SUBFAMILY F2* (*CYP81F2*) is involved in glucosinolate metabolism, and its loss-of-function mutants show impaired resistance to fungal attack (Bednarek et al., 2009; Hunziker et al., 2020). *ETHYLENE RESPONSIVE FACTOR 6* (*ERF6*) encodes a central regulator of stress-induced inhibition of plant growth that is involved in the response to reactive oxygen species (Li et al., 2025; Sewelam et al., 2013). RT-qPCR analysis detected lower expression of these three marker genes in the three *stt3a* mutant lines compared to WT following treatment with Pi-Cer D for 1 h or 3 h (Figure 10A). Among the mutant lines, *stt3a-4* showed intermediate expression between WT and the *stt3a-3* and *stt3a-5* mutants. The *stt3a-2* T-DNA insertion line also showed compromised induction of *PEN2*, *CYP81F2*, and *ERF6* expression in response to the same treatment (Figure 11A).

I also investigated MAPK activation in WT and the three mutants following the treatment of seedlings with Pi-Cer D (Figure 10B). WT seedlings exhibited clear MAPK activation at 30 min after the onset of treatment. By contrast, all mutants exhibited diminished MAPK activation. MAPK activation patterns mirrored the transcriptional responses, with *stt3a-4* showing an intermediate phenotype. Similarly, the *stt3a-2* T-DNA insertion line showed lower MAPK activation compared to Col-0 (Figure 11B). As a negative control, the *rda2-3* mutant exhibited no induction of marker gene expression and no MAPK activation.

I also observed lower expression of the marker genes and lower MAPK activation when I used 9Me-Spd as the elicitor instead of Pi-Cer D (Figure 12; Figure 13).

STT3A* contributes to resistance against the fungal pathogen *Colletotrichum higginsianum

The lower expression of defense-related genes and the diminished activation of MAPK in the *stt3a* mutants suggested that *STT3A* is required for plant defense against pathogens. To test this hypothesis, I inoculated WT, *rda2-5*, harboring a mutation in the kinase domain of RDA2 and showing insensitive phenotype to Pi-Cer D treatment (Kato et al., 2022), *ncer2-2*, *stt3a-3* (L-08), *stt3a-4* (L-13), *stt3a-5* (L-51), and *pad3-1* (susceptible control; Glazebrook and Ausubel, 1994), which lacks PHYTOALEXIN DEFICIENT 3 function, with a fungus *Colletotrichum higginsianum*, which is a hemibiotrophic pathogen infecting Arabidopsis. Filamentous fungi generally contain sphingolipids with 9-methyl sphingoid bases (Huang et al., 2019; Koga et al., 1998; Ramamoorthy et al., 2009). The *stt3a-3*, *stt3a-4*, and *stt3a-5* mutants were more susceptible to *C. higginsianum* than WT, as evidenced by the size of lesions developing on leaves (Figure 14). Furthermore, the *stt3a* mutants were more susceptible to *C. higginsianum* than *rda2-5* and *ncer2-2* (Figure 14). Similar results were observed in T-DNA insertion lines (*rda2-3*, *ncer2*, and *stt3a-2*) (Figure 15). These results suggest that *STT3A* affects the defense of Arabidopsis plants against fungal infection.

***STT3A* affects the molecular size of NCER2**

STT3A is involved in the post-translational modification of proteins (Koiwa et al., 2003). I determined that the reduction in the bioluminescence response in the *stt3a* mutants was more pronounced in response to Pi-Cer D vs. 9Me-Spd treatment (Figure 4C; Figure 5), suggesting that *STT3A* exerts a greater influence on NCER2 than RDA2.

The neutral ceramidase of fruit fly (*Drosophila melanogaster*) undergoes glycosylation, a common modification involved in protein secretion and stability (Yoshimura et al., 2022). Therefore, I focused on the abundance and molecular size of NCER2 in the WT and *stt3a* mutants. NCER2 comprises two distinct structural units; when the N-terminal neutral/alkaline non-lysosomal ceramidase domain is cleaved from the C-terminal portion of the protein, the two resulting fragments form a complex (Kato et al., 2022). I performed immunoblotting with an antibody that recognizes the C-terminus of NCER2 (anti-NCER2) in WT, the *stt3a* mutants (*stt3a-3* and *stt3a-5*), and a *ncer2* mutant (*ncer2-2*; Kato et al., 2022), using total protein extracts and apoplast wash fluid (AWF), which should be enriched in secreted proteins (Figure 16A). In WT, I detected a single band (of ~50 kDa) corresponding to the expected molecular size of the C-terminus of NCER2, whereas in *ncer2-2*, no band was observed. By contrast, I observed the downward band shift of NCER2 in *stt3a-3* and *stt3a-5*, indicating a difference in the molecular mass of NCER2. Furthermore, I observed that the band shift was restored in the *stt3a-3* plants complemented with wild-type *STT3A* (Figure 16B). These results suggest that NCER2 is regulated by *STT3A*-mediated protein modification. To confirm whether NCER2 is subject to N-glycosylation, I treated the deglycosylation enzymes, Endo H, PNGase A, and PNGase F to AWF extracted from WT. The downward band shift was observed in PNGase A-treated AWF (Figure 16C), indicating that NCER2 undergoes N-glycosylation which can be removed by PNGase A.

***STT3A* is required for the modification of RDA2.**

To investigate whether *STT3A* is also involved in the modification of RDA2, I generated *stt3a-3* mutant expressing RDA2 fused with an HA epitope tag (RDA2-HA)

(Figure 17A). RDA2-HA complemented the *rda2-4* mutant phenotype, restoring Pi-Cer D-triggered bioluminescence (Kato et al., 2022; Figure 17B), indicating that the HA tag does not interfere with RDA2 function. Immunoblot analysis of the T₁ transformants expressing RDA2-HA revealed a downward shift in band size in the *stt3a-3* background compared to WT (Figure 17C), suggesting that the loss of *STT3A* alters RDA2 modification. In order to identify whether RDA2 is subject to N-glycosylation, the extracted proteins were treated with a deglycosylation enzyme, PNGase A and subsequently subjected to immunoblotting. After PNGase A treatment, both WT and *stt3a-3* samples expressing RDA2-HA showed the same lower molecular size band (Figure 17D). In contrast, the untreated *stt3a-3* sample displayed a band of intermediate size—larger than the PNGase A-treated band but smaller than the untreated WT band. These results demonstrate that RDA2 is modified by N-glycosylation which is targeted by PNGase A, and *STT3A* may contribute to the part of its N-glycosylation.

DISCUSSION

Our Lumi-Map analysis revealed that *STT3A* is required for defense responses in Arabidopsis following the recognition of sphingolipids (Figure 4; Figure 6; Figure 7). *STT3A* is involved in protein N-glycosylation, which supports proper protein folding and function. For example, *STT3A*-mediated N-glycosylation is important for the function of cellulose biosynthesis enzymes (Kang et al., 2008). In Arabidopsis *stt3a* mutants, defects in N-glycosylation result in insufficient glycosylation of β -glucosidase, an enzyme responsible for converting conjugated forms of the phytohormones abscisic acid and auxin into their active forms (Jiao et al., 2020a). *STT3A* also participates in temperature sensitivity, salinity tolerance, and responses to abiotic stress through increased stomatal density (Jiao et al., 2020b; Liu et al., 2018; Rips et al., 2014; Zhang et al., 2009).

Although all three mutants analyzed harbor mutations in *STT3A*, their phenotypes in reporter assays and their defense responses differed (Figure 4; Figure 5). The *stt3a-3* and *stt3a-5* mutants showed the weakest defense responses, whereas *stt3a-4* exhibited intermediate defense responses compared to WT (Figure 4; Figure 10). Since *stt3a-3* and *stt3a-4* are predicted to affect splicing, *STT3A* transcripts in these two mutants might be different from those of WT. *STT3A* in *stt3a-5* harbors a G616D mutation that is likely located in the catalytic domain or the dolichol-binding lipid-exposed groove, both of which are essential for oligosaccharyltransferase activity, thus potentially impairing N-glycosylation efficiency (Niu et al., 2020; Wild et al., 2018). Therefore, the mutation in *stt3a-5* might inhibit the enzymatic activity of *STT3A* or affect its stability.

NCER2 is a ceramidase located in the plant apoplast that converts microbial-derived ceramides into sphingoid bases (Kato et al., 2022). I observed a band shift for C-terminus

of NCER2 in apoplast fluid collected from the *stt3a* mutants (Figure 16), suggesting that NCER2 is post-translationally modified by *STT3A*. Our results suggest that NCER2 could be a target of *STT3A*-mediated N-glycosylation. Deglycosylation of NCER2 from WT plants caused a band shift only with PNGase A, but not with PNGase F or Endo H (Figure 16C). Since PNGase A, unlike PNGase F and Endo H, cleaves N-glycans even when $\alpha(1,3)$ -linked core fucose is present (Wang et al., 2024), NCER2 is likely modified with complex-type N-glycans containing such residues. This result supports the hypothesis that NCER2 undergoes N-glycosylation and that this process may depend on *STT3A* function. Future studies should identify precise NCER2 glycosylation sites using LC-MS/MS-based glycoproteomics, validate their importance through site-directed mutagenesis, and determine how these modifications influence NCER2 stability and enzymatic activity.

From the perspective of plant immunity, *STT3A* is known to maintain the stability and function of plasma membrane-localized PRRs including EFR and leucine-rich repeat receptor-like kinases (LRR-RLKs) via their N-glycosylation (Häweker et al., 2010; Saijo et al., 2009). In this study, *WRKY33* transcription in the three *stt3a* mutants was also insensitive to treatment with elf18 (Figure 4), confirming a role for *STT3A* in EFR regulation (Häweker et al., 2010; Saijo et al., 2009). Moreover, in experiments with various elicitors, the *stt3a* mutants showed less pronounced responses to 9Me-Spd and COS (Figure 5). Cellulose-derived COS, along with xylooligosaccharides (XOS), are recognized by leucine-rich repeat-malectin receptor kinases such as IMPAIRED GLYCAN PERCEPTION 1 (IGP1, also reported as CELLOOLIGOMER-RECEPTOR KINASE 1 [CORK1]), IGP3, and IGP4 (Fernández-Calvo et al., 2024; Pring et al., 2023; Tseng et al., 2022); these receptors may also be targets of modification by *STT3A*. Our

findings extend the role of *STT3A* in maintaining the modification of the lectin receptor RDA2. Our immunoblot analysis revealed that RDA2-HA expressed in the *stt3a-3* mutant had a lower molecular size compared to RDA2-HA expressed in WT, and that PNGase A treatment of WT samples expressing RDA2-HA caused a band shift to the same position as that observed in *stt3a-3* expressing RDA2-HA (Figures 17C and 17D). These results indicate that the loss of *STT3A* function affects the modification of RDA2, which may be related to impaired N-glycosylation. Furthermore, the downward band shift between untreated and PNGase A-treated *stt3a-3* samples expressing RDA2-HA suggests that its glycosylation may occur through *STT3A*-independent mechanisms (Figure 17D). Further studies including a mutational analysis of predicted N-glycosylation sites would elucidate how N-glycosylation regulates RDA2 function.

The *stt3a* mutants were less resistant to the fungal pathogen *C. higginsianum* than WT (Figure 14). *C. higginsianum* contains sphingolipids including 9Me-Spd; their recognition by RDA2 might be important for plant defense against this pathogen. The lower expression of *PEN2* and *CYP81F2* in the *stt3a* mutants following sphingolipid treatment (Figure 10; Figure 12) may result in the compromised production and accumulation of antimicrobial compounds, leading to the lower resistance against filamentous fungi (Figure 14). The *stt3a* mutants exhibited markedly increased susceptibility to *C. higginsianum* compared to *rda2* and *ncer2* mutant plants (Figure 14; Figure 15). This difference suggests that in addition to signaling mediated by NCER2 and RDA2, multiple pathways for the recognition of PAMPs and damage-associated molecular patterns are affected by mutations in *STT3A* (Figure 4; Figure 5).

In this study, I demonstrated that *STT3A* is required for the proper modification of both NCER2 and RDA2, the two key components of sphingolipid-triggered immunity.

These findings suggest that *STT3A* plays a broader role in the recognition of sphingolipids involved in plant immunity. In future, comparative glycoproteomic analyses between WT and *stt3a* mutant plants will be valuable to identify PTI-related proteins that require N-glycosylation.

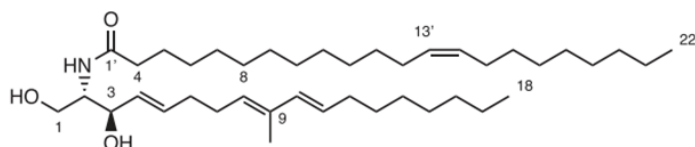
Table 1. Candidate genes and polymorphisms identified in the high SNP-index intervals on Arabidopsis chromosome 5.

Mutant line	Chromosome coordinate	Reference base	Altered base	Depth	SNP-index	Accession number	Note	Type of mutation
L-08	chr5_5447710	G	A	31	0.96	AT5G16610	hypothetical protein	S52L
	chr5_5685154	G	A	27	0.96	AT5G17270	Protein prenyltransferase superfamily protein	junction
	chr5_5853828	G	A	40	0.95	AT5G17730	P-loop containing nucleoside triphosphate hydrolases superfamily	L58F
	chr5_6180512	G	A	29	1	AT5G18590	Galactose oxidase/kelch repeat superfamily	A238V
	chr5_6233885	G	A	19	1	AT5G18690	arabinogalactan protein 25	P81L
	chr5_6283053	G	A	12	1	AT5G18840	Major facilitator superfamily protein	E34K
	chr5_6654303	G	A	31	0.96	AT5G19690	STT3A	junction
	chr5_7143069	G	A	20	0.9	AT5G21030	PAZ domain-containing protein	L281F
	chr5_7150195	G	A	28	1	AT5G21060	Glyceraldehyde-3-phosphate dehydrogenase-like	R245*
	chr5_7350638	G	A	32	1	AT5G22170	transmembrane protein	G22R
L-13	chr5_4830408	G	A	20	1	AT5G14930	SAG101	G418E
	chr5_5853107	G	A	17	1	AT5G17730	P-loop containing nucleoside triphosphate hydrolases superfamily	S298F
	chr5_6057819	G	A	15	1	AT5G18290	SIP1;2, SIP1B, SMALL AND BASIC INTRINSIC PROTEIN 1B	G16D
	chr5_6656475	G	A	11	1	AT5G19690	STT3A	junction
L-51	chr5_5466541	G	A	42	1	AT5G16660	Low-density receptor-like protein	S18F
	chr5_5524628	G	A	34	1	AT5G16800	N-ACETYLTRANSFERASE 60, NAA60	H232Y
	chr5_5727773	G	A	28	1	AT5G17390	Adenine nucleotide alpha hydrolases-like superfamily protein	G45I
	chr5_5750510	G	A	32	1	AT5G17440	LETHAL UNLESS CBC 7 B, LUC7B	D45N
	chr5_5938597	G	A	26	1	AT5G17920	METS1	G599S
	chr5_6155575	G	A	27	1	AT5G18540	E3 ubiquitin-protein ligase	D116N
	chr5_6195241	G	A	24	1	AT5G18610	PBL27	R471K
	chr5_6415323	G	A	18	1	AT5G19120	Eukaryotic aspartyl protease family protein	G247R
	chr5_6457379	G	A	29	1	AT5G19190	hypothetical protein	V11I
	chr5_6657197	G	A	26	1	AT5G19690	STT3A	G616D
	chr5_6729528	G	A	27	1	AT5G19900	PRL1-interacting factor	P173L

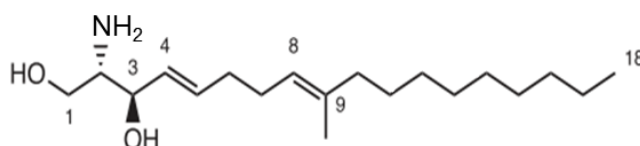
Table 2. Primers used in this study.

Name	Sequence (5'-3')	Description
WRKY33_qPCR_F	CTCAAGCACCATATACATTCA	For RT-qPCR
WRKY33_qPCR_R	CCTTTGCTCTAGAGAATCCACC	
PEN2_qPCR_F	TCAGGTAAATCAGTTCGAATCAAGAAC	
PEN2_qPCR_R	TGAGGAAACCTGTTGGAGAAAGGATC	
CYP81F2_qPCR_F	ATTGTCCGCATGGTCACAGGGAG	
CYP81F2_qPCR_R	GTAGCCGTGTCCGAACACTTTAAG	
ERF6_qPCR_F	TCGAATCCTCCTCGCGTTACTG	
ERF6_qPCR_R	TTCGGTGGTGCGATCTTCAACG	
STT3A_qPCR_F	TATGATCGGGTGAGGCGGAC	
STT3A_qPCR_R	TGTTCTGGGAGGTTTCAGC	
UBC_qPCR_F	CTGCGACTCAGGGAATCTTCTAA	
UBC_qPCR_R	TTGTGCCATTGAATTGAACCC	
rda2-3_RP	TGCAATCATTTTTGGGAAGTG	For genotyping
rda2-3_LP	TACTCCGGTGACATATAGCCG	
stt3a-2_RP	TCAATGTTTGGCTTTCTTGG	
stt3a-2_LP	CAAAGACGTGTAGGCCATCAC	
ncer2_RP	TGCATACTTAATCGGAGTCGG	
ncer2_LP	ATTTACGCTTCGAACCGTTTC	
LBb1.3_BP	ATTTTGCCGATTTCGGAAC	For complementation vector
STT3A_comp_F	ATGCCTGCAGGTCGATTGGTATAACAGAGTG TTCCAAGC	
STT3A_comp_R	GATCGGGGAAATTCGGACGTCCATGAGATGTA CTTTAGTG	

Pi-Cer D



9Me-Spd
(4*E*,8*E*)-9-methyl-
4,8-sphingadienine



9Me-Spt
(4*E*,8*E*,10*E*)-9-
methyl-4,8,10-
sphingatrienine

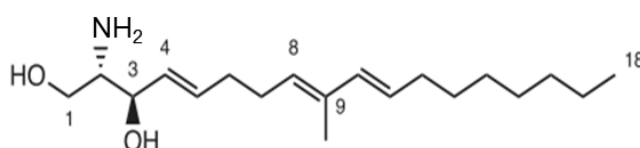


Figure 1. Chemical structures of Pi-Cer D, (4*E*,8*E*)-9-methyl-4,8-sphingadienine (9Me-Spd), and (4*E*,8*E*,10*E*)-9-methyl-4,8,10-sphingatrienine (9Me-Spt) sphingolipids. Pi-Cer D have been isolated from *Phytophthora infestans*, an oomycete pathogen.

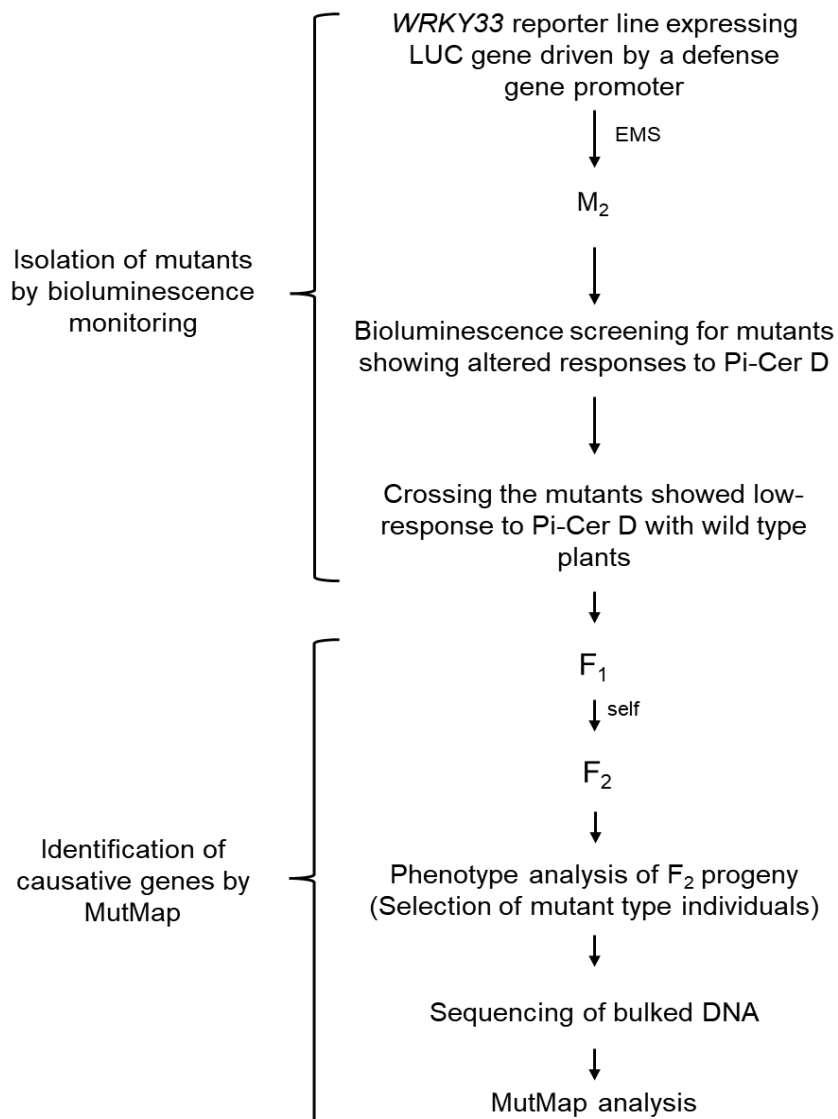
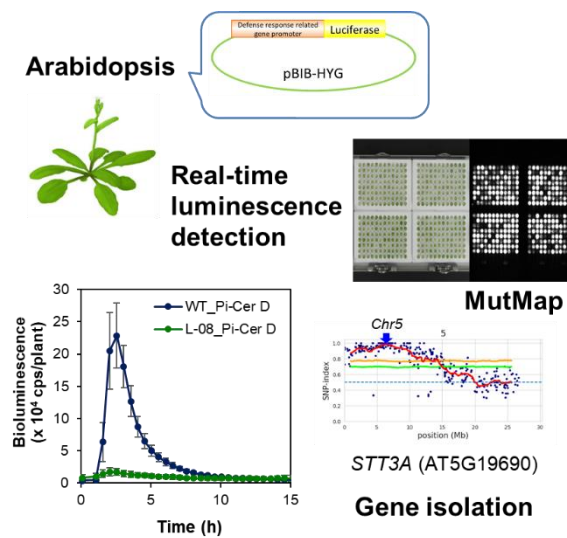


Figure 2. Lumi-Map method used in the study for identification of genes involved in Pi-Cer D perception and defense signaling. Top: A simplified scheme of the Lumi-Map pipeline. Bottom: Detailed procedure of Lumi-Map used in the study. The mutants were generated by EMS mutation of the *WRKY33-LUC* reporter line. The mutants were crossed with the *WRKY33-LUC* reporter line, and the F₁ population was subsequently selfed to generate the F₂ population. The F₂ population was screened with a real-time bioluminescence monitoring system. The bulked DNA was made from individuals of the F₂ population showing low responses to Pi-Cer D treatment, and was used for MutMap analysis to identify the causative genetic changes responsible for the mutant phenotype.

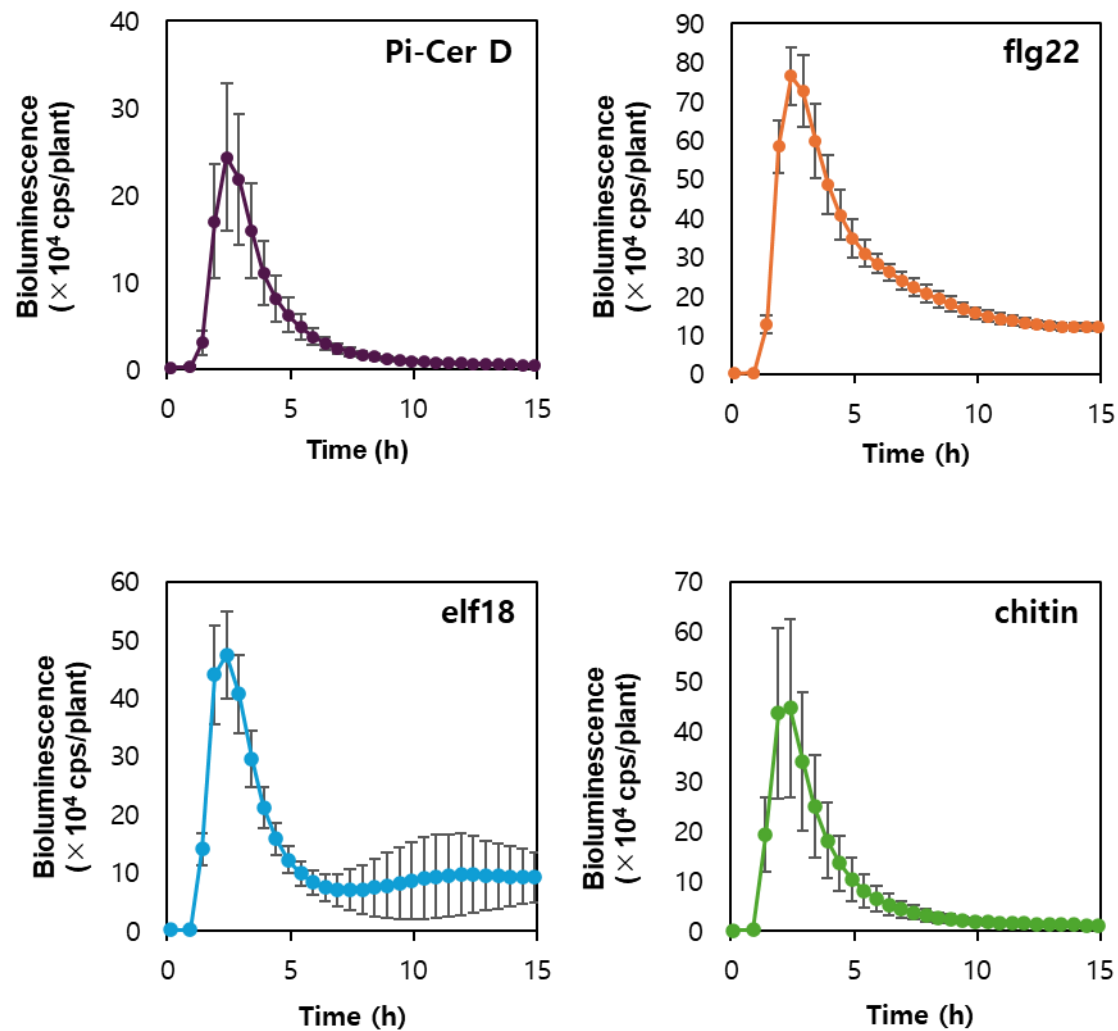


Figure 3. Luciferase-mediated bioluminescence patterns of *WRKY33-LUC* reporter plants after treatment of each PAMP. Seven-day-old seedlings were treated with Pi-Cer D (0.17 μ M), flg22 (0.1 μ M), elf18 (0.1 μ M), or chitin 20 μ g/ml). The bioluminescence of each seedling was monitored at the indicated time points using a real-time bioluminescence monitoring system. The variants reach their peak between 0 and 5 hours of post-PAMP treatment, after which they begin to decline in response.

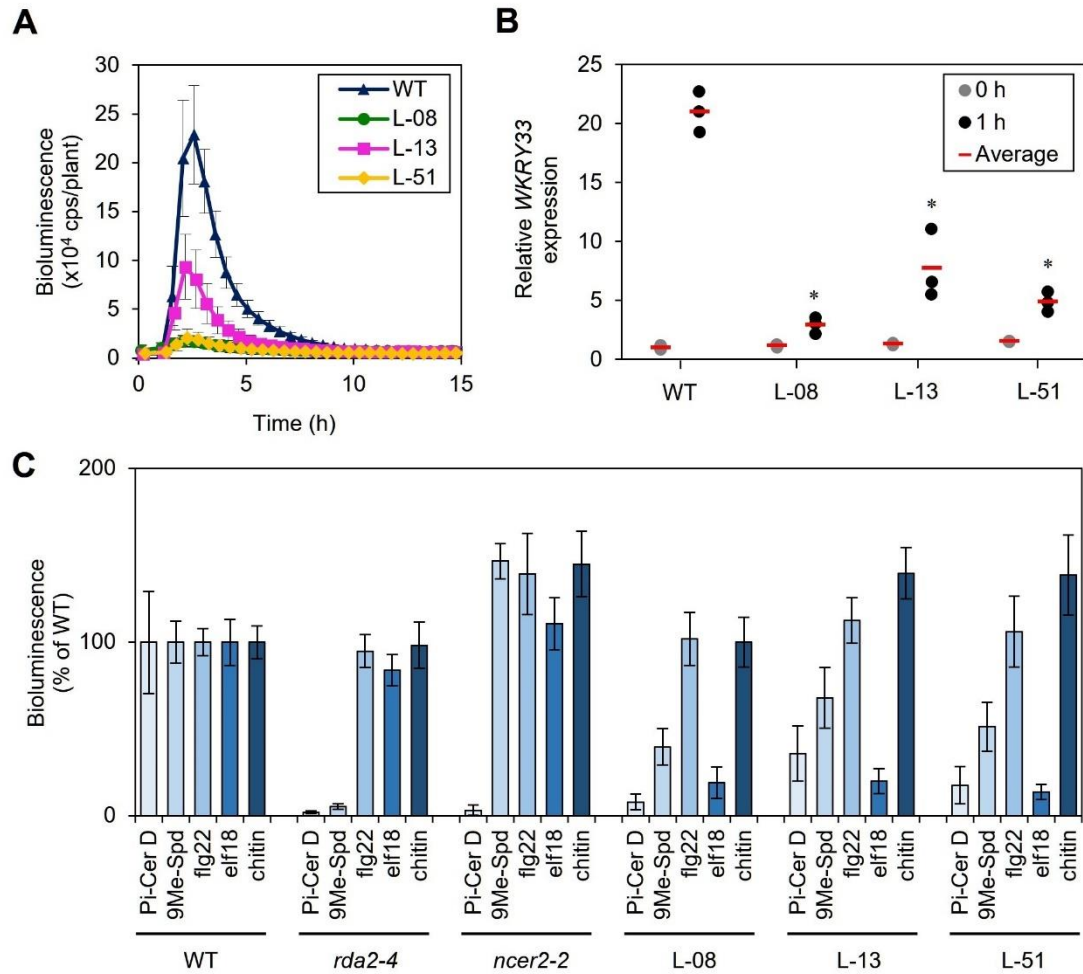


Figure 4. Isolation and characterization of mutants with impaired responses to Pi-Cer D. (A) Bioluminescence patterns of the three identified mutants under Pi-Cer D treatment (means $\pm$ SE). Seven-day-old wild type (*pWRKY33-LUC* reporter line; WT), L-08, L-13, and L-51 mutant seedlings were treated with 0.17 μ M Pi-Cer D. The bioluminescence of each seedling was monitored at the indicated time points starting at the onset of treatment using a continuous bioluminescence monitoring system. (B) Relative *WRKY33* expression levels in response to Pi-Cer D treatment in WT and the three mutants, as determined by RT-qPCR. *WRKY33* expression levels were measured in seven-day-old seedlings treated with 0.17 μ M Pi-Cer D. *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for WT at each time point. (C) Bioluminescence responses of WT and the five mutants following treatment with five elicitors (means $\pm$ SE). Seven-day-old seedlings were treated with 0.17 μ M Pi-Cer D, 0.5 μ M (4E,8E)-9-methyl-4,8-sphingadienine (9Me-Spd), 0.1 μ M flg22, 0.1 μ M elf18, or 20 μ g/ml chitin, and the bioluminescence of each seedling was monitored. The bioluminescence level in WT was set to 100%, to which the bioluminescence levels in the mutants were normalized.

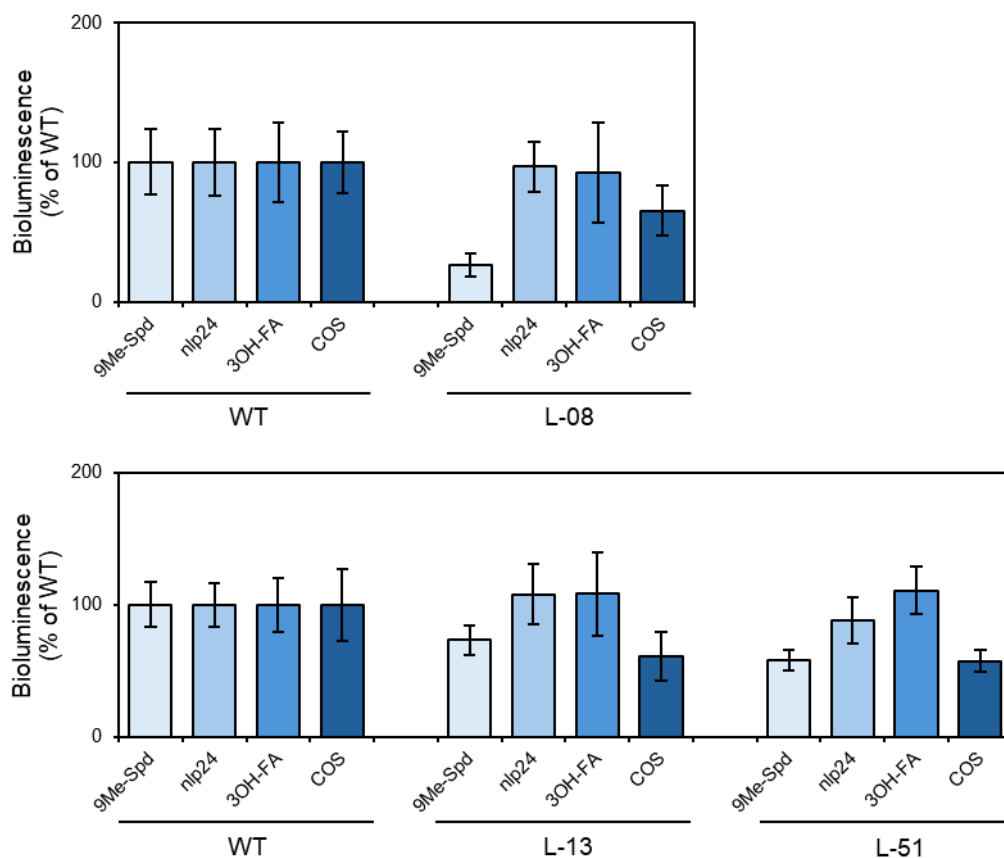


Figure 5. Bioluminescence responses of the three mutants, L-08, L-13, and L-51 to treatment with four elicitors. Seven-day-old wild type (*pWRKY33-LUC* reporter line; WT), L-08, L-13, and L-51 seedlings were treated with 0.5 μ M (4E,8E)-9-methyl-4,8-sphingadienine (9Me-Spd), 0.1 μ M nlp24, 1 μ M 3-hydroxy fatty acid (3-OH-FA), or 20 μ g/ml cello-oligosaccharides (COS). The bioluminescence level of the WT was set to 100%, to which the bioluminescence levels in the mutants were normalized.

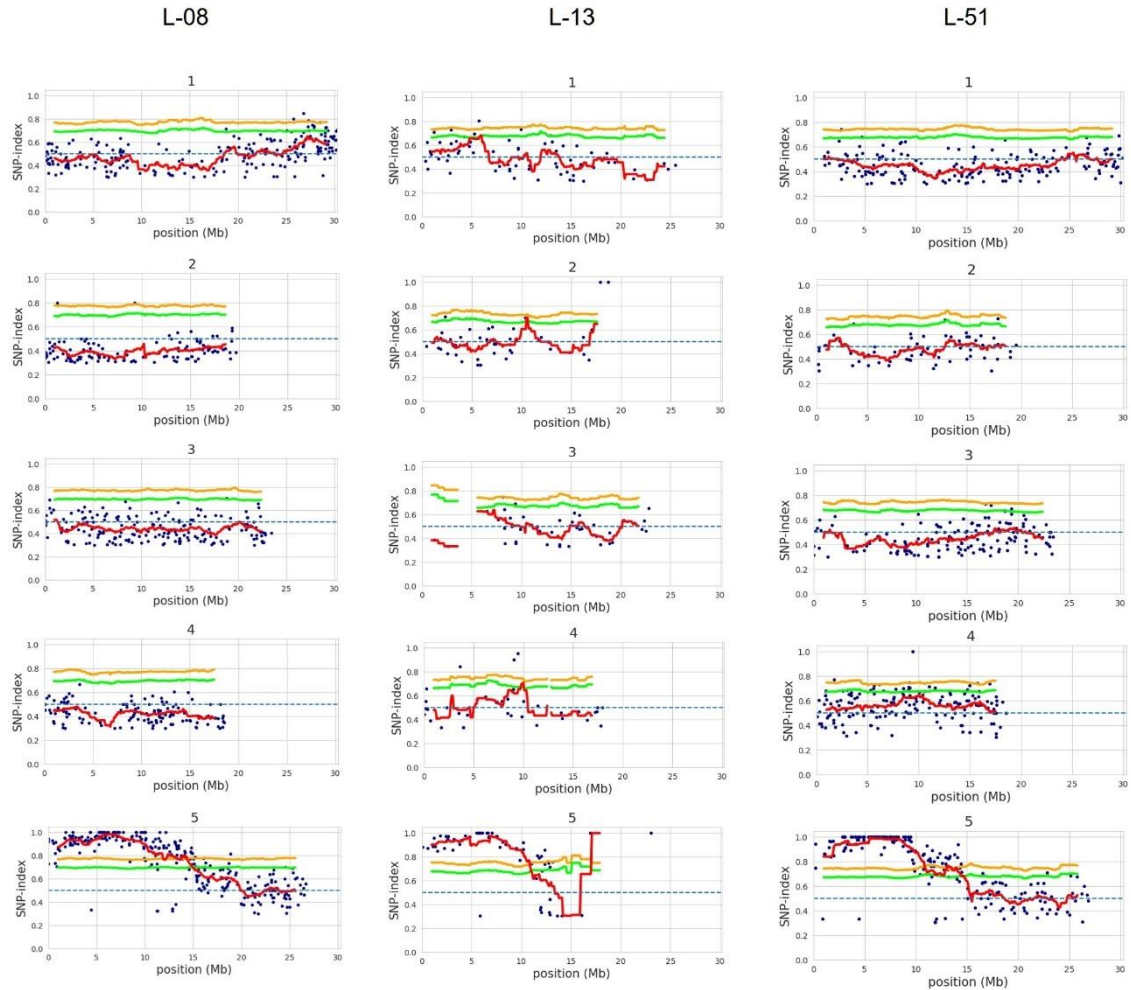


Figure 6. MutMap analysis of the L-08, L-13, and L-51 mutants. The highest SNP-index peak within a genomic region indicates the location of the causative mutation. An SNP-index peak around the 6-Mb region on chromosome 5 was observed in all three mutants: L-08, L-13, and L-51. Blue dots represent individual mutations in each mutant. The red line represents average SNP-index values across a 2-Mb sliding window with 10-kb increments. The green and yellow lines indicate the 95% and 99% confidence limits, respectively, of SNP-index values under the null hypothesis of an SNP-index = 0.5 assuming no linkage between the SNP and the causal mutation.

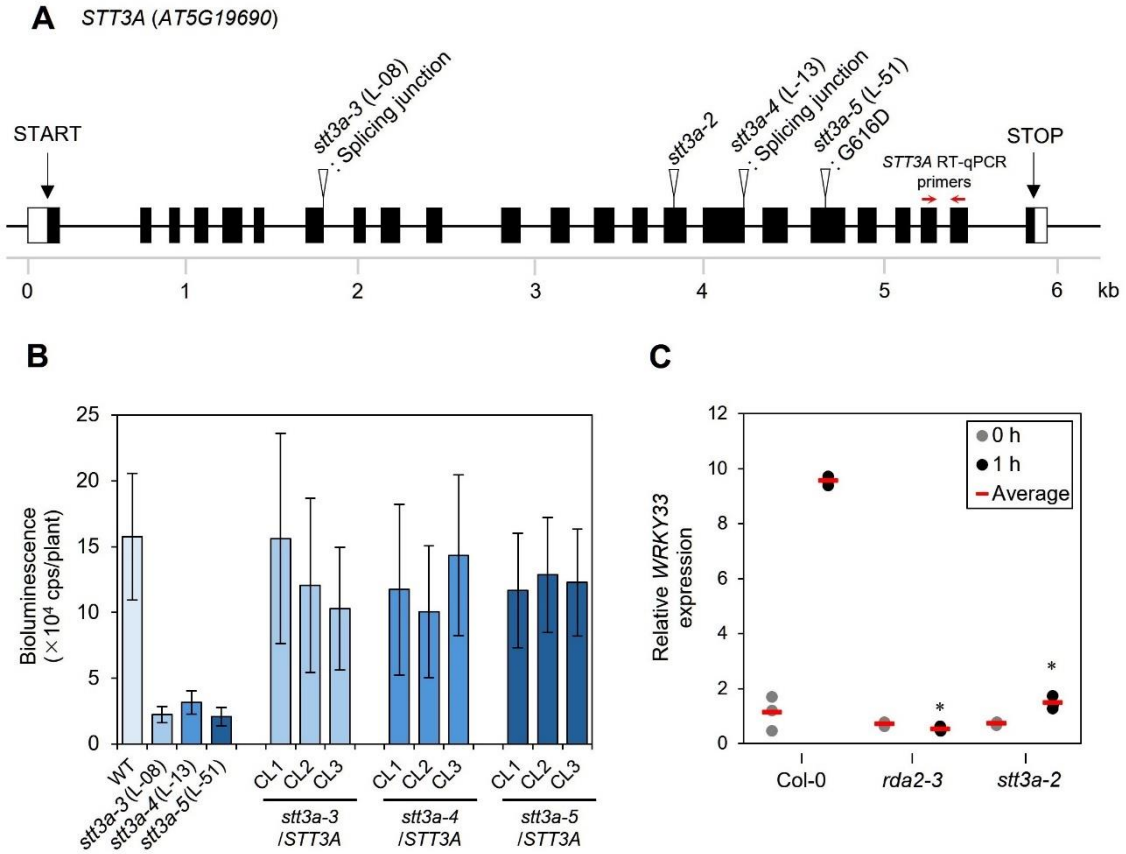


Figure 7. *STT3A* is involved in the recognition of Pi-Cer D in plants. (A) *STT3A* gene structure and the locations of the causative mutations in the three mutants, *stt3a-3* (L-08), *stt3a-4* (L-13), and *stt3a-5* (L-51). Black rectangles and white rectangles indicate exons and UTRs respectively; lines represent introns. The locations of causative mutations are represented by white triangles, with their mutation types indicated. The red arrows indicate the primer positions for RT-qPCR with *STT3A*. (B) Genetic complementation of the three mutants with a wild-type of the *STT3A* gene. The wild-type *STT3A* gene was introduced into each mutant background (10.7-kb genomic fragment with its native promoter). The p*WRKY33-LUC* reporter line (WT), the L-08, L-13, L-51 mutants, and complementation lines for each mutant were treated with 0.17 μ M Pi-Cer D (means $\pm$ SE); bioluminescence was monitored using a continuous bioluminescence monitoring system. (C) Relative *WRKY33* expression levels in Col-0 and the T-DNA insertion lines *stt3a-2* and *rda2-3*, as determined by RT-qPCR. Seven-day-old seedlings were used for the analysis. The seedlings were sampled at 0 and 1 h of treatment with 0.17 μ M Pi-Cer D. *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for Col-0 at each time point.

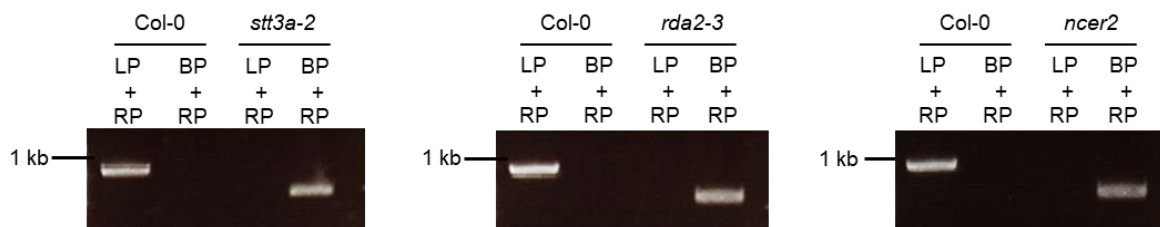


Figure 8. Confirmation of the T-DNA insertion in the *stt3a-2*, *rda2-3*, and *ncer2* mutant lines. Gel electrophoresis of the PCR products confirmed the genotypes of the T-DNA insertion lines and Col-0. LP and RP indicate the left primer and right primer of the respective gene, respectively; BP indicates the T-DNA border primer.

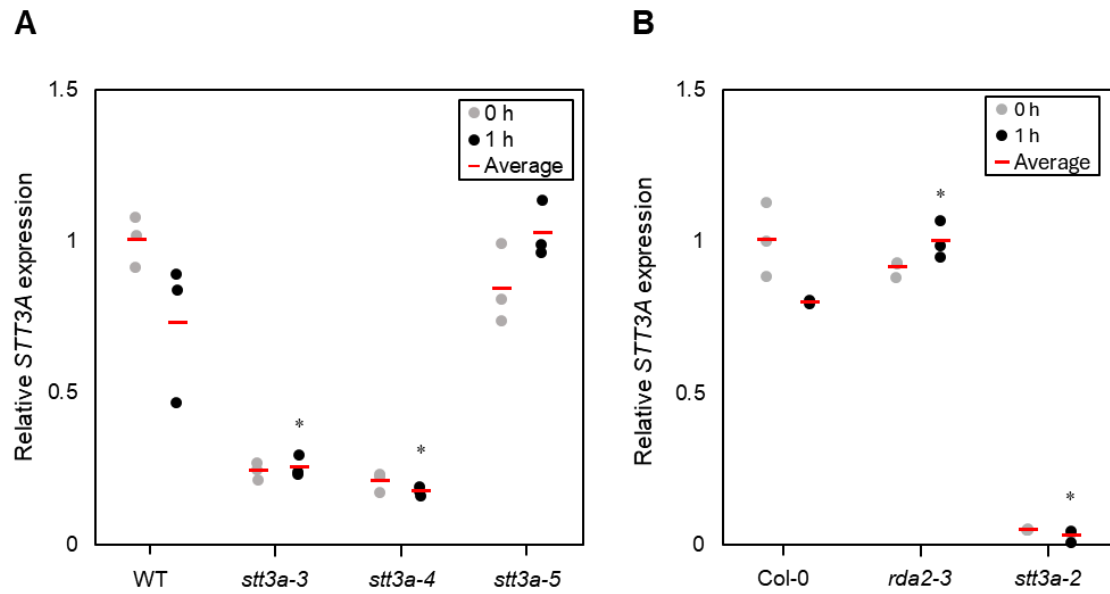


Figure 9. Relative expression levels of the *STT3A* gene in (A) *stt3a* mutants and (B) T-DNA insertion lines. Seven-day-old seedlings of the *pWRKY33-LUC* reporter line (WT), *stt3a-3*, *stt3a-4*, *stt3a-5*, Col-0, *rda2-3*, and *stt3a-2* were treated with 0.17 μ M Pi-Cer D. The seedlings were sampled at 0 and 1 h after treatment ($n = 3$) (means $\pm$ SE). *, $p < 0.05$ in two-tailed t -tests compared with the corresponding values for WT or Col-0 at each time point.

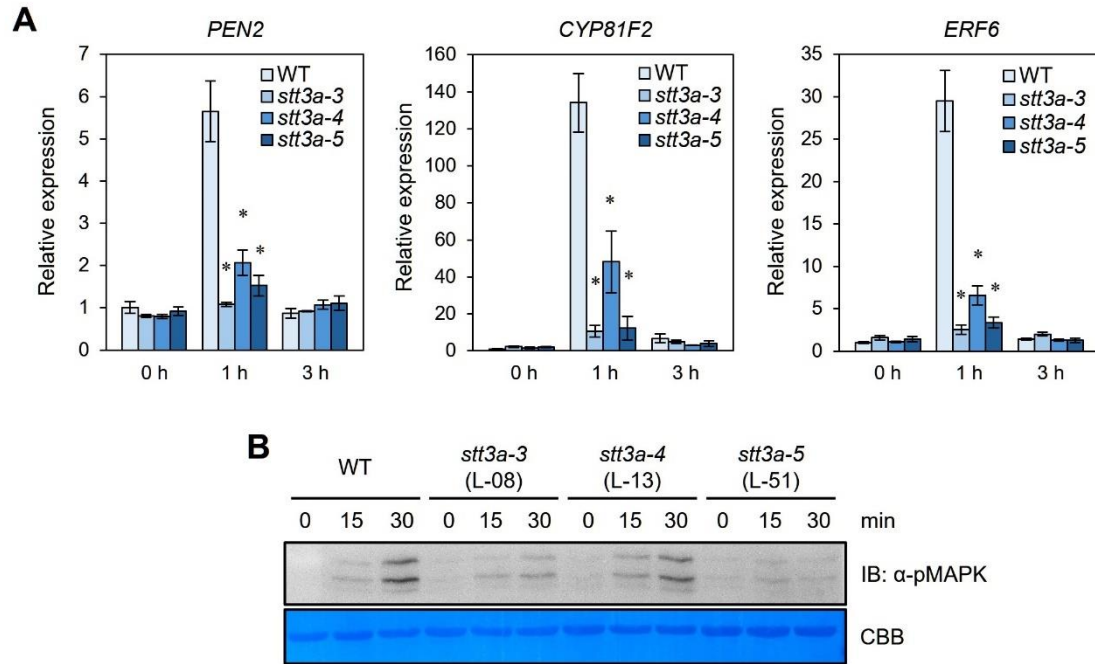


Figure 10. *STT3A* is required for the defense response following the recognition of Pi-Cer D. (A) Relative expression levels of the defense-related genes *PEN2*, *CYP81F2*, and *ERF6* as determined by RT-qPCR (means $\pm$ SE). Seven-day-old seedlings of the p*WRKY33-LUC* reporter line (WT), *stt3a-3* (L-08), *stt3a-4* (L-13), and *stt3a-5* (L-51) were treated with 0.17 μ M Pi-Cer D. The seedlings were sampled at 0, 1, and 3 h after treatment ($n = 3$). *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for WT at each time point. (B) MAPK phosphorylation assay in WT and the three *stt3a* mutants. Seven-day-old seedlings were sampled at 0, 15, or 30 min of treatment with 0.17 μ M Pi-Cer D. Phosphorylated MAPKs were visualized with anti-phospho-p44/p42 MAPK antibody. Equal protein loading was determined by staining the membrane with Coomassie brilliant blue (CBB).

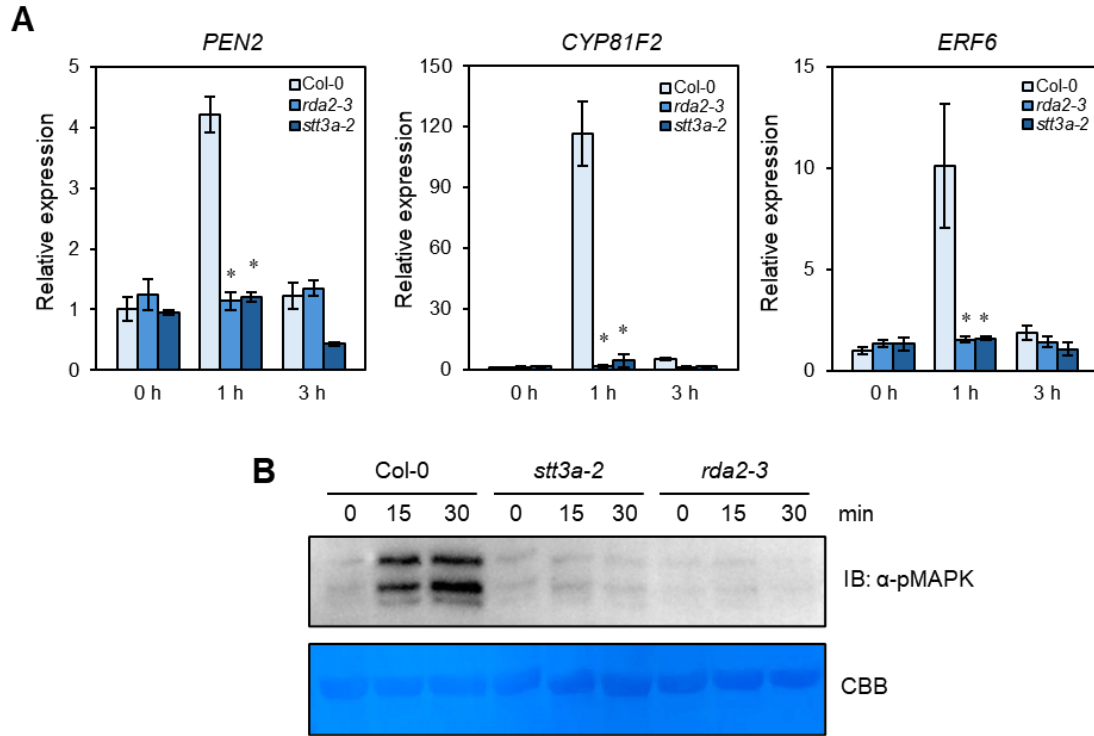


Figure 11. *STT3A* is required for defense responses in the *stt3a* T-DNA insertion mutant following Pi-Cer D treatment. (A) Relative expression levels of the defense-related genes *PEN2*, *CYP81F2*, and *ERF6* as determined by RT-qPCR. Seven-day-old Col-0, *stt3a-2*, and *rda2-3* seedlings were treated with 0.17 μ M Pi-Cer D, and sampled at 0, 1, or 3 h after treatment ($n = 3$). *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for Col-0 at each time point. (B) MAPK phosphorylation assay in Col-0, *stt3a-2*, and *rda2-3*. Seven-day-old seedlings were sampled at 0, 15, or 30 min of treatment with 0.17 μ M Pi-Cer D. Phosphorylated MAPKs were visualized with anti-phospho-p44/p42 MAPK antibody. Equal protein loading was determined by staining the membrane with Coomassie brilliant blue (CBB).

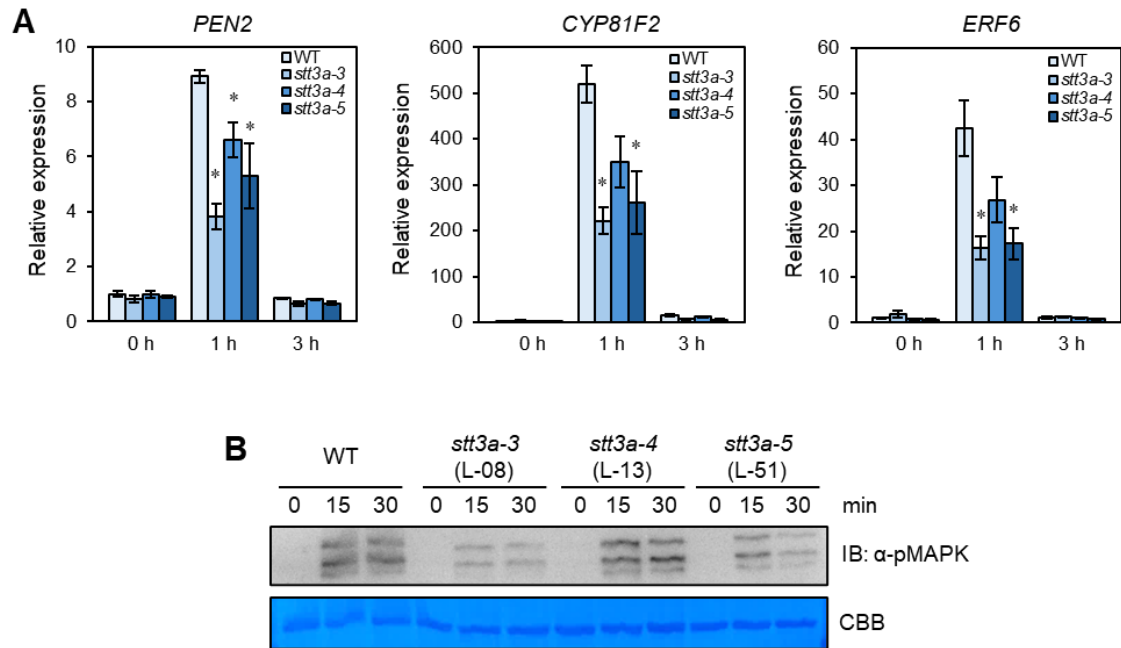


Figure 12. *STT3A* is required for defense responses in the *stt3a* mutants following 9Me-Spd treatment. (A) Relative expression levels of the defense-related genes *PEN2*, *CYP81F2*, and *ERF6* as determined by RT-qPCR (means $\pm$ SE). The p*WRKY33-LUC* reporter line (WT) and each *stt3a* mutant were treated with 0.5 μ M 9Me-Spd. Seven-day-old seedlings were sampled at 0, 1, or 3 h of treatment with 9Me-Spd ($n = 3$). *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for WT at each time point. (B) MAPK phosphorylation assay in WT and each *stt3a* mutant. Seven-day-old seedlings were sampled at 0, 15, and 30 min of treatment with 0.5 μ M 9Me-Spd. Phosphorylated MAPKs were visualized with anti-phospho-p44/p42 MAPK antibody. Equal protein loading was determined by staining the membrane with CBB.

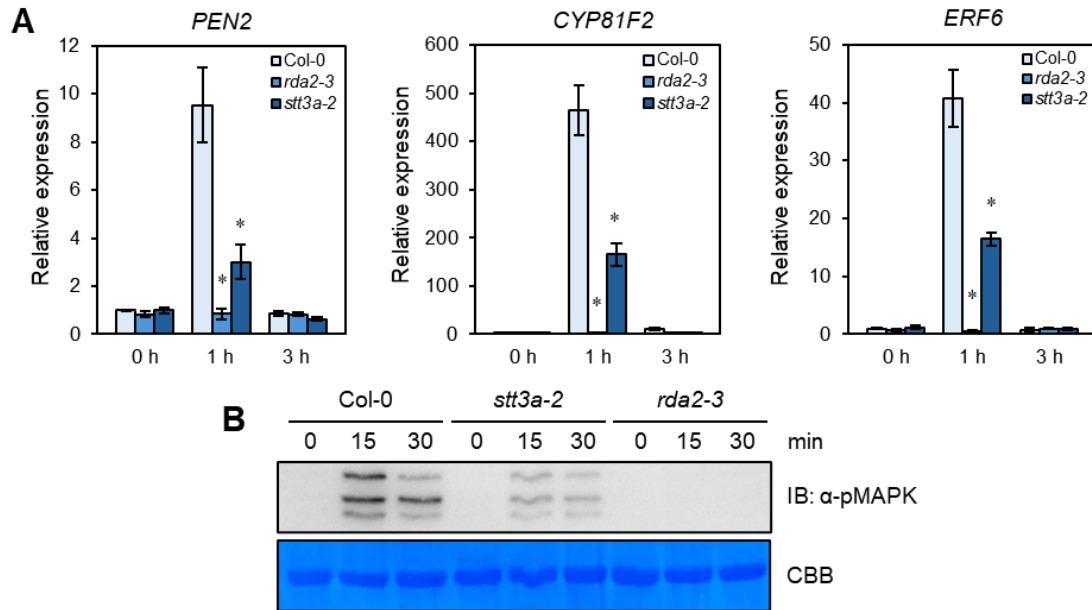


Figure 13. *STT3A* is required for defense responses in the *STT3A* T-DNA insertion line after 9Me-Spd treatment. (A) Relative expression levels of the defense-related genes *PEN2*, *CYP81F2*, and *ERF6* as determined by RT-qPCR. Col-0, the *STT3A* T-DNA insertion line *stt3a-2*, and *rda2-3* as a control were treated with 0.5 μ M 9Me-Spd. Seven-day-old seedlings were sampled at 0, 1, or 3 h of treatment with 9Me-Spd ($n = 3$). *, $p < 0.01$ in two-tailed t -tests compared with the corresponding values for Col-0 at each time point. (B) MAPK phosphorylation assay in Col-0, *stt3a-2*, and *rda2-3*. Seven-day-old seedlings were sampled at 0, 15, and 30 min of treatment with 0.5 μ M 9Me-Spd. Equal protein loading was determined by staining the membrane with CBB.

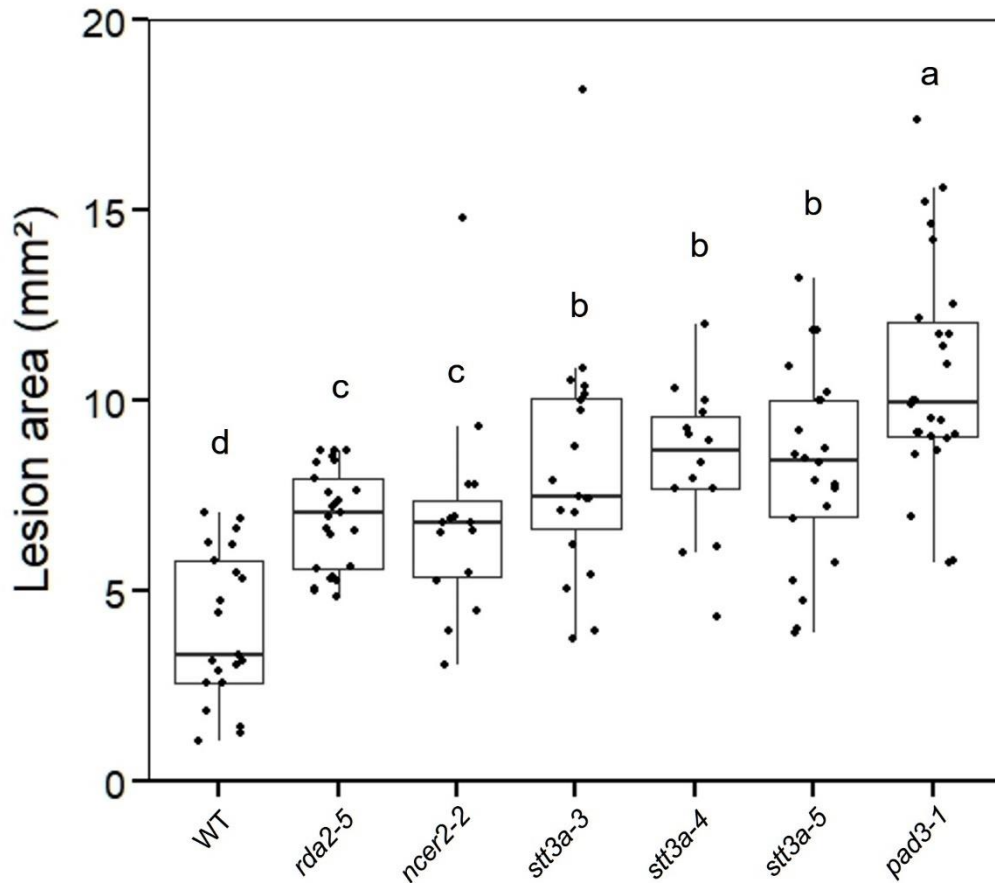


Figure 14. The *stt3a* mutants show enhanced susceptibility to *Colletotrichum higginsianum*. Six-week-old plants of the pWRKY33-LUC reporter line (WT), *rda2-5*, *ncer2-2*, *stt3a-3*, *stt3a-4*, *stt3a-5*, and *pad3-1* were inoculated with a *C. higginsianum* spore suspension containing 1.5×10^5 conidiospores/ml. Boxplots showing lesion area in WT, *rda2-5*, *ncer2-2*, the *stt3a* mutants and *pad3-1*. Lesion size was measured 4 days after inoculation. Box bounds represent the lesion area, center line represents the median, and whiskers indicate the range of the maximum or minimum data. Different lowercase letters represent statistically significant differences ($p < 0.05$), as revealed by Tukey's test. The results were replicated in two separate experiments.

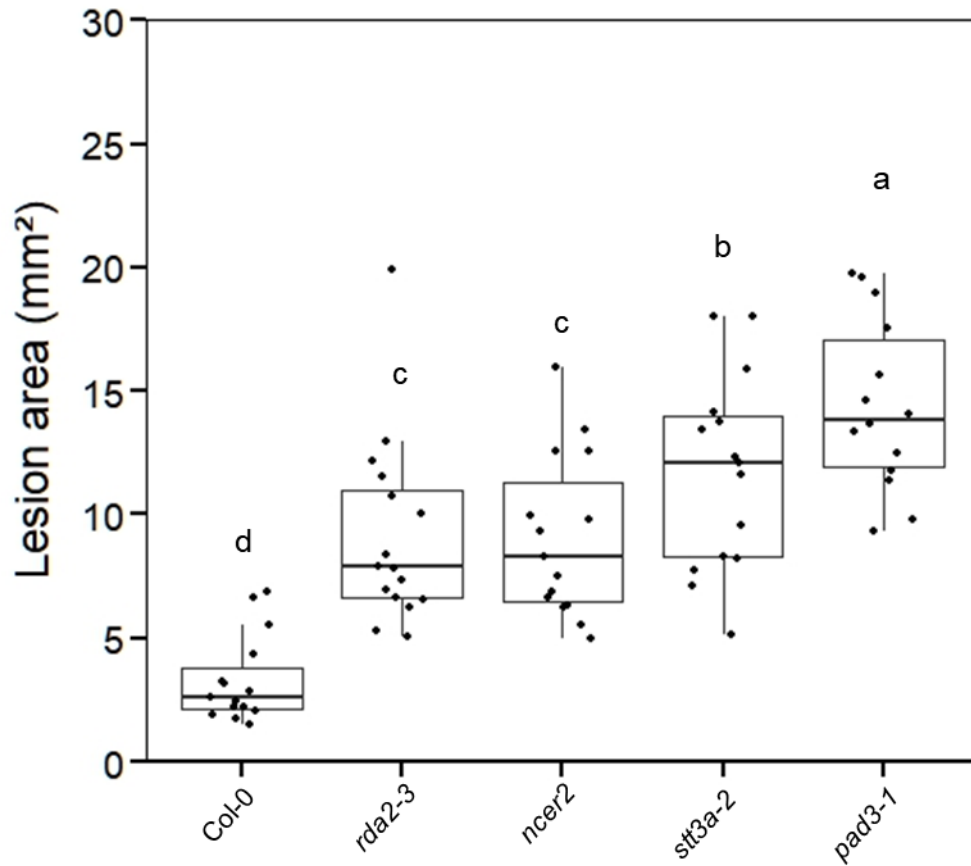


Figure 15. The *stt3a* T-DNA insertion line shows enhanced susceptibility to *C. higginsianum*. Six-week-old plants of Col-0, *rda2-3*, *ncer2*, *stt3a-2*, and *pad3-1* were inoculated with a *C. higginsianum* spore suspension containing 1.5×10^5 conidiospores/ml. Boxplots showing lesion area in Col-0, *rda2-3*, *ncer2*, *stt3a-2*, and *pad3-1*. Lesion size was measured 4 days after inoculation. Box bounds represent the lesion area, center line represents the median, and whiskers indicate the range of the maximum or minimum data. Different lowercase letters represent statistically significant differences ($p < 0.05$), as revealed by Tukey's test. The results were replicated in two separate experiments.

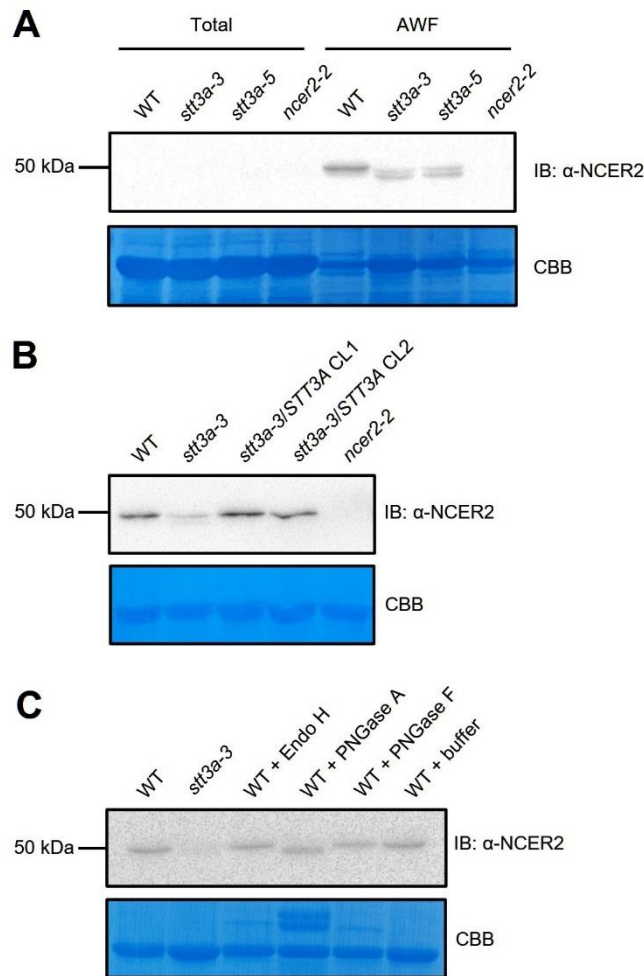


Figure 16. *STT3A* is required for modification of NCER2. (A) The downward band shift occurred in *stt3a* mutants. Immunoblot analysis of NCER2 protein in total protein extracts and apoplast wash fluid (AWF) from five-week-old plants of the *pWRKY33-LUC* reporter line (WT), *stt3a-3*, *stt3a-5*, and *ncer2-2*. Proteins were detected using an anti-NCER2 antibody recognizing the C-terminal region. A band corresponding to the expected molecular size of NCER2 was observed in WT, but a downward shifted band was detected in the *stt3a* mutants, while no band was detected in the *ncer2-2*. The results were replicated in three separate experiments. (B) The band shift of NCER2 was restored to the WT pattern in *stt3a-3* complementation lines (*stt3a-3/STT3A* CL1, *stt3a-3/STT3A* CL2). Five-week-old plants were used for immunoblotting of AWF in WT and the *stt3a-3*, *stt3a-3* complemented lines, and *ncer2-2*. NCER2 was detected with a specific antibody against NCER2. The results were replicated in three separate experiments. (C) NCER2 is modified by N-glycosylation. WT AWF was treated with deglycosylation enzymes, Endo H, PNGase A, or PNGase F, followed by immunoblotting with anti-NCER2 antibody. Five-week-old plants were used for immunoblotting of AWF in WT and the *stt3a-3*. The results were replicated in two separate experiments.

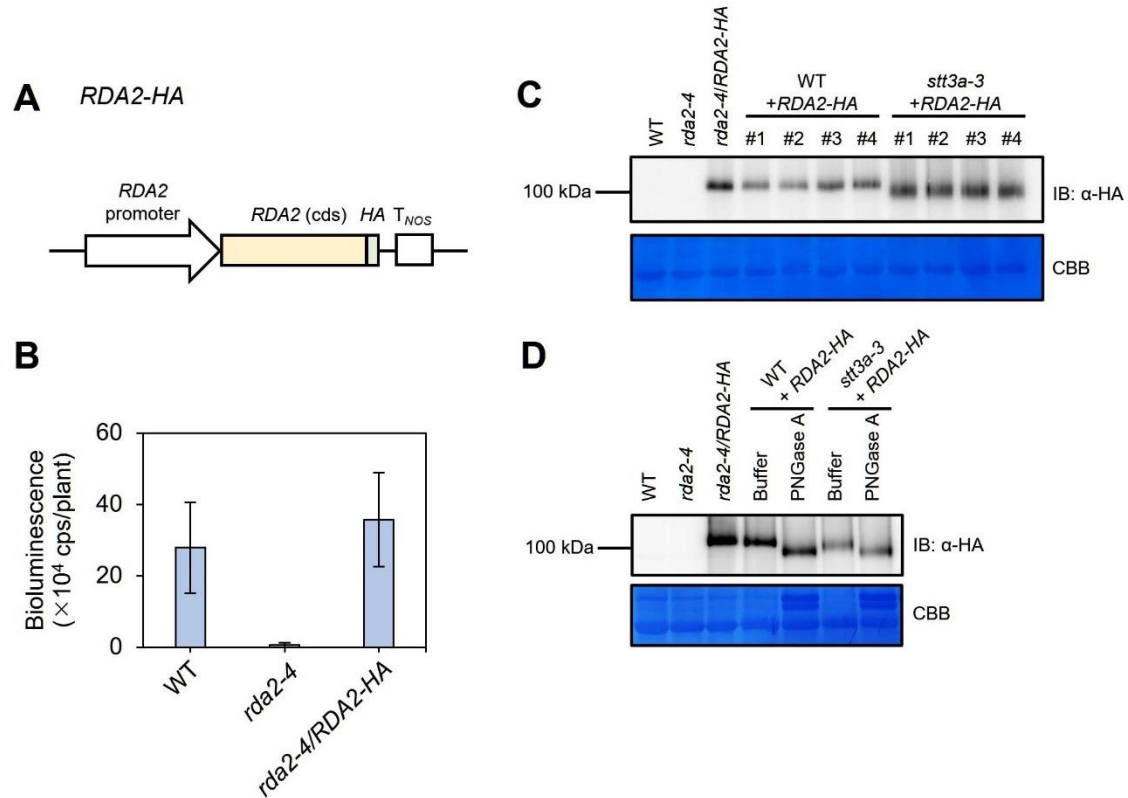


Figure 17. *STT3A* is required for modification of RDA2. (A) Schematic representation of the *RDA2-HA* construct. (B) Bioluminescence analysis of *rda2-4/RDA2-HA* line with *pWRKY33-LUC* reporter line (WT). Seven-day-old seedlings were treated with 0.17 μ M Pi-Cer D (means $\pm$ SE). (C) The downward band shift was observed in *stt3a-3* expressing *RDA2-HA*. Immunoblot analysis of *RDA2-HA* modification in the WT and *stt3a-3* mutant backgrounds using anti-HA antibody. Total proteins were extracted from four-week-old *T₁* transgenic plants. The results were replicated in three separate experiments. (D) *RDA2* is modified by N-glycosylation. Total protein extracts from WT and *stt3a* mutant plants expressing *RDA2-HA* were treated with or without the deglycosylation enzyme, PNGase A prior to SDS-PAGE. The results were replicated in three separate experiments.

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SUMMARY

Plants recognize invading pathogens through an innate immune system that detects conserved pathogen-associated molecular patterns (PAMPs). While receptors for protein and carbohydrate PAMPs are well characterized, far less is known about how plants perceive pathogen-derived lipids, particularly sphingolipids produced by fungi and oomycetes. These sphingolipids often contain a 9-methyl-branched sphingoid base that is absent in plants, making it a distinctive non-self molecular signature. Understanding how plants recognize these lipid molecules is essential for clarifying an underexplored branch of pattern-triggered immunity (PTI).

In *Arabidopsis thaliana*, the ceramide Pi-Cer D from the oomycete *Phytophthora infestans* functions as a lipid-based PAMP. Pi-Cer D is processed in the apoplast by the ceramidase NEUTRAL CERAMIDASE 2 (NCER2), generating a 9-methyl-branched sphingoid base that is subsequently recognized by the plasma membrane receptor RESISTANT TO DFPM-INHIBITION OF ABSCISIC ACID SIGNALING 2 (RDA2), a lectin receptor-like kinase. This two-step mechanism establishes a foundation for sphingolipid-triggered immunity, but the full set of molecular components required for this pathway has not been defined. In particular, it is unknown how plants ensure the proper maturation, stability, and function of the proteins involved in sphingolipid perception.

The aim of this dissertation was to identify additional components required for Pi-Cer D recognition and to investigate how protein glycosylation contributes to sphingolipid-triggered immune signaling. To achieve this, I used Lumi-Map, a luciferase-based forward genetic screening platform that enables rapid detection of mutants with

impaired defense gene activation. Screening for individuals with reduced *WRKY33-LUC* induction after Pi-Cer D treatment led to the identification of mutations in *STT3A*, the catalytic subunit of the oligosaccharyltransferase (OST) complex responsible for co-translational N-glycosylation in the endoplasmic reticulum.

Characterization of *stt3a* mutants revealed that *STT3A* is essential for a full immune response to Pi-Cer D. Loss of *STT3A* resulted in reduced *WRKY33* expression and impaired MAPK activation following sphingolipid treatment. These defects were selective: responses to several peptide or carbohydrate PAMPs were less affected, indicating that sphingolipid-triggered PTI relies particularly strongly on *STT3A* function.

Biochemical analyses showed that both NCER2 and RDA2 depend on *STT3A* for proper glycosylation and stable accumulation. In *stt3a* mutants, NCER2 exhibited altered glycosylation patterns and reduced protein abundance, and RDA2 showed decreased accumulation and shifts in molecular size consistent with glycosylation defects. These findings indicate that *STT3A*-mediated N-glycosylation is required for correct folding, processing, and receptor function in the Pi-Cer D perception pathway.

Consistent with impaired sphingolipid recognition, *stt3a* mutants displayed enhanced susceptibility to the fungal pathogen *Colletotrichum higginsianum*, demonstrating the physiological relevance of this pathway for disease resistance. Together, these results identify *STT3A* as a previously unrecognized but critical component of sphingolipid-triggered immunity and establish a functional link between N-glycosylation and lipid-based PAMP perception.

Overall, this dissertation provides new insight into how plants detect pathogen-derived sphingolipids and highlights the importance of ER protein maturation in enabling this form of immune recognition. These findings broaden the conceptual framework of

PTI and suggest that future studies of plant lipid receptors should consider the essential role of glycosylation in their biogenesis and function.

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