

Exploration of plant recognition receptors as tools to engineer crop immunity

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1 Introduction

1.1 Plant immunity - The zig-zag model

Plants are constantly attacked by microbes, herbivores, nematodes, and parasite plants in the natural environment. Unlike mammals that can utilize multilayered somatic adaptive immune systems, plants solely rely on individual cell immunity against the invasion of bacteria, fungi, oomycetes, and viruses. In 1989, it was the first time that the basic principle of immunity, pattern recognition theory, was presented by Charles A. Janeway, which is the best paper that has never published. Charles A. Janeway integrated two types of immunity containing innate immunity and adaptive immunity in mammals and coined the term pattern recognition receptors which can recognize non-self pathogen-associated molecular patterns (PAMPs) and subsequently arouse innate immunity (Medzhitov, 2009). Based on this theory and research, in 2006, the plant immune system was described as a zigzag model (Jones & Dangl, 2006). In this model, plants employ two layers of detection systems to fend off pathogens which are called PTI and ETI, 1) First barrier: Cell surface-localized pattern recognition receptors (PRRs) perceive PAMPs derived from non-adapted microbes or endogenous damage-associated molecular patterns (DAMPs) to activate pattern-triggered immunity (PTI) which confers basal resistance. However, tricky pathogens won't lay down arms and surrender, the successful ones will deploy virulent effectors to overcome PTI resulting in effector-triggered susceptibility (ETS); 2) Second barrier: As the cell surface receptors fall in battle, the intracellular nucleotide-binding (NB) leucine-rich-repeat (LRR) domain receptors (NLRs) keep fighting with virulence effectors secreted by adapted microbes directly or indirectly, termed effector-triggered immunity (ETI). Recurrent cycles of recognition, escaping, and perception actuate plant-pathogen co-evolution and researchers' curiosity about the discovery and characterization of ambiguous distinction between PTI and ETI.

1.1.1 Pattern Recognition Receptors (PRRs) in PTI

In plants PRRs comprise receptor-like kinases (RLKs) and receptor-like proteins (RLPs) which are defined according to the presence or absence of the intracellular kinase domain (Albert et al., 2020). In 1994, the first plasma membrane localized receptor *Cf-9*, which recognizes *Avr9* from the leaf mold fungus *Cladosporium fulvum*, was identified in tomato (Jones et al., 1994). Since 2000, the accomplishment of genome sequence analysis in the model plant *Arabidopsis thaliana* (The Arabidopsis Genome, 2000) and comprehensive molecular genetic approaches facilitated the discovery of numerous PRRs (Boutrot & Zipfel, 2017). In recent years, PRRs and corresponding elicitor ligands in different plant species have been characterized (W. L. Wan et al., 2019).

1.1.1.1 PRRs involved in bacterial PAMP perception

The first identified well-studied PRR in *Arabidopsis thaliana* (*Arabidopsis* later) is FLS2 (LRR-RLK) which binds a conserved N-terminal 22-amino acid peptide (flg22) of bacterial flagellin and is wide-spread in plants (Chinchilla et al., 2006; Gómez-Gómez, 2000; Monaghan & Zipfel, 2012). Another motif in flagellin (flgII-28) distinct from flg22 is recognized by FLS3 in Solanaceae plants (Hind et al., 2016). Another well-known bacterial receptor EFR (LRR-RLK) which recognizes bacterial elongation factor Tu (EF-Tu) epitope elf18 is Brassicaceae specific (Zipfel et al., 2006). Without flagellin, the bacteria *Ralstonia solanacearum* still induces immune response in *Arabidopsis* (Pfund et al., 2004). The identification of RLP32 as the receptor which confers perception of bacterial translation-initiation factor 1 (IF1), proving the existence of additional receptors sensing bacterial PAMPs in *Arabidopsis* (Fan et al., 2022). Xup25, from xanthine/uracil permease in *Pseudomonas syringae*, is perceived by the RLK XPS1 (xanthine/uracil permease sensing 1) to activate immune response (Mott et al., 2016). Not only peptides, other components like lipid 3-hydroxydecanoic acid from lipopolysaccharide in Gram-negative bacteria, is perceived through the lectin S-domain receptor kinase LORE (lipooligosaccharide-specific reduced elicitation) (Kutschera et al., 2019; Ranf et al., 2015). Peptidoglycans (PGNs) are derived from bacterial cell walls and perceived by a complex formed by LYM1, LYM3, and CERK1 which are lysin-motif domain proteins and kinase (Willmann et al., 2011). In other plant species, receptors have been identified such as OsXA21 (LRR-RLK) in rice recognizes RaxX16 derived from *Xanthomonas* RaxX protein and SICORE in tomato senses csp22 from bacterial cold shock protein (Pruitt et al., 2015; L. Wang et al., 2016).

1.1.1.2 PRRs involved in fungal PAMP recognition

Many fungal PAMPs and paired receptors involved in PTI have been identified in recent years. In *Arabidopsis*, RLP30 perceives SCP^{Ss} full-length protein from the fungal pathogen *Sclerotinia sclerotiorum*, and RLP42 recognizes pg9 derived from *Botrytis cinerea* endopolygalacturonases (PGs), respectively. Distinct epitopes from those proteins are shown to be recognized by other sensors in Brassicaceae species (Yang et al., 2023; Zhang et al., 2021; Zhang et al., 2014; Zhang et al., 2013). Chitin is a common component in fungal cell walls and acts as a PAMP which is perceived by a LysM-RK complex comprising LYK4, LYK5 and CERK1 (Cao et al., 2014; Miya et al., 2007; Wan et al., 2012). The perception of fungal ethylene-inducing xylanase (EIX) in tomato is mediated by LRR-RLP SIEIX1 and SIEIX2, both of which can bind EIX, but only SIEIX2 can trigger defense responses (Ron & Avni, 2004).

1.1.1.3 PRRs involved in oomycete perception

Via PRRs, plants sense effectors conserved in oomycete with PAMP features leading to defense response. Elicitins (INF1) secreted by *Phytophthora* species are recognized by LRR-

RLP receptors SmELR in *Solanum microdontum* and NbREL in *Nicotiana benthamiana* which cause cell death (Chen et al., 2023; Du et al., 2015). Pep-13, a conserved peptide derived from *Phytophthora* transglutaminases triggers cell death in potato, is dependent on the recognition by LRR-RLK PERU (Lin et al., 2022; Torres Ascurra et al., 2023). *N. benthamiana* LRR-RLP NbrXEG1, an ortholog of SIEIX1 and SIEIX2, is specifically required for recognition of xyloglucan-specific endoglucanase XEG1 rather than elicitor EIX (Wang et al., 2018). A conserved peptide nlp20 derived from bacteria, fungi and oomycetes NLPs is sensed by RLP23 to trigger immune responses in Arabidopsis (Albert et al., 2015).

1.1.2 NLR receptors involved in ETI

NLRs consist of a conserved tripartite architecture: an N-terminal domain, a central nucleotide-binding (NB) domain, and a C-terminal leucine-rich repeat (LRR) domain. NB domains serve as a molecular switch to operate signal state from inactive ADP-bound to active ATP-bound (Bernoux et al., 2016), LRR domains function to associate with another two domains to mediate NLRs auto-inhibition, or with directly binding effectors for ligand recognition (Krasileva et al., 2010; Qi et al., 2012). The N-terminal domain is sufficient to activate downstream signaling and immunity activation as a signal domain (Wang et al., 2015).

NLRs in angiosperms and gymnosperms are divided into three main classes based on the N-terminal domains: the coiled-coil (CC) NLRs (CNLs), Toll/Interleukin-1 receptor/Resistance (TIR) protein NLRs (TNLs), and RPW8-like CC domain (RPW8) NLRs (RNLs). CNLs and TNLs are considered as sensor NLRs while RNLs and some CNLs as helper NLRs. In addition to these subclades, there are other NRC (NLR Required for Cell Death) helper NLRs which are firstly reported as a signaling converter for cell surface receptors and intracellular receptors in *Solanum lycopersicum*, then found to be involved in the Pto-mediated cell death in *N. benthamiana* (Gabriels et al., 2007; Sueldo et al., 2015; Wu et al., 2016). Different from CNLs that exist in monocots and dicots, TNLs are only found in dicots, demonstrating the distinct evolution of NLRs and providing CNL resources for breeding in monocots (Jubic et al., 2019).

Some NLRs recognize effectors directly by physical interaction between the LRR domain or ID (integrated domain) and the cognate effectors. The TNL receptor RPP1 from Arabidopsis binds to its effector ATR1 from the oomycete *Hyaloperonospora arabidopsidis* in an LRR-ligand binding manner which triggers a complex of four RPP1 monomers (Krasileva et al., 2010; Ma et al., 2020). Sensor NLR with ID targeted by effectors works with helper NLR and transduces signal leading to recognition and cell death. In Arabidopsis RRS1 and RPS4 form a heterodimeric complex, the WRKY domain of RRS1 is directly acetylated by PopP2 effector from bacteria *Ralstonia solanacearum* leading to RPS4-mediated immunity. In inactive state,

RRS1 negatively regulates RPS4 activation to inhibit the auto-active cell death caused by helper RPS4 (Deslandes et al., 2003; Le Roux et al., 2015; Li et al., 2015; Williams et al., 2014).

Most NLRs perceive the host intermediate changes modified by effectors. There are two models explaining such indirect interactions: one is the guard model, NLRs (guard protein) perceive the interference of host protein (guardee) functions by effectors directly; another one is the decoy model, in which NLRs monitor the modification of decoys which mimic the effector target without other function in immunity (van der Hoorn & Kamoun, 2008). For example, Arabidopsis RIN4 is a “guardee” targeted by many effectors like AvrRPM1 or AvrB, and AvrRpt2 from *P. syringae* which activate guard CNL RPM1, RPS2 via ADP-ribosylating or phosphorylating activities, and cleaving RIN4, respectively (Chung et al., 2014; Mackey et al., 2003; Mackey et al., 2002; Xu et al., 2017).

In animals, it has been demonstrated that the oligomerization of NLRs is essential for its function (Hu et al., 2013). In plants, the resistosome of ZAR1-RKS1-PBL2^{UMP} in Arabidopsis was identified and functionally studied. In the inactive state, the CNL ZAR1 binds ADP and interacts with RKS1. After AvrAC uridylylating decoy protein PBL2, the active ZAR1-RKS1 forms a complex with PBL2 accompanied by exchanging ATP for ADP, which promotes five heterotrimers forming a pentameric wheel-like structure termed resistosome. The ZAR1 resistosome acts as a calcium channel in plasma membrane by pricking the PM leading to cell death (Bi et al., 2021; Wang et al., 2019). The oligomers and resistosomes formation results in generation of small TIRs hydrolysate molecules and replacement of ADP to ATP, linking up downstream signaling and immune outputs (El Kasmi, 2021).

TNL TIR domains have NADase catalytic activity, which is able to cleave NAD⁺ after oligomer arrangement. The small variant-cyclic-ADP-ribose products are supposed to be signal molecules and activate downstream modules (Locci et al., 2023; L. Wan et al., 2019). Two modules are required for downstream signaling of immunity activation in Arabidopsis: EDS1-PAD4-ADR1 is for TNL- and CNL-triggered ETI and PRR-mediated PTI, the EDS1-SAG101-NRG1 is for TNL-triggered ETI. In *N. benthamiana*, EDS1-SAG101-NRG1 module mediates TNL-triggered ETI, however, EDS1-PAD4-ADR1 is required for stomatal immunity in both *N. benthamiana* and Arabidopsis (Wang et al., 2023). The heterodimer EDS1-PAD4 binds 5'-phosphoribosyl-ADP/AMP released from NAD⁺ cleavage by TNLs resulting in recruitment of ADR1, while EDS1-SAG101 binds ADPr-ATP produced by TNLs catalysis leading to interaction with NRG1. ADR1 and NRG1 form resistosomes serving as Ca²⁺ channels at the plasma membrane (Feehan et al., 2023; Huang et al., 2022; Jia et al., 2022). As helper NLRs, AtADR1 participates in TNL- and CNL-mediated SA (salicylic acid) immunity pathway, whereas AtNRG1 is only involved in TNL-triggered cell death (Cui et al., 2017;

Lapin et al., 2019; Sun et al., 2021). In Solanaceous plants, another type of helper NLRs, NRCs, build signaling networks with sensor NLRs. For example, upon activation of the sensor by pathogen effectors, NRCs oligomerize and form PM-associated resistosomes to initiate cell death (Ahn et al., 2023; Contreras et al., 2023; Kourelis et al., 2022).

1.2 PRR co-receptors and downstream signaling implicated in plant immunity

1.2.1 PRR co-receptors

Unlike LRR-RKs, for LRR-RPs it's crucial to form a receptors/co-receptors complex in the absence of the cytoplasmic kinase domain for signal cascade transmission. LRR-RPs form a ligand-independent constitutive complex with co-receptor LRR-RK SOBIR1 to possess equal function compared to authentic LRR-RKs and recruit co-receptor SERK3/BAK1 in a ligand-dependent manner (Gust & Felix, 2014). LRR-RK BAK1, belongs to the SOMATIC-EMBRYOGENESISRECEPTOR-LIKE KINASE (SERK) family and functions with BRI1 (LRR-RK) which is involved in regulating brassinosteroid signaling (Li et al., 2002). In addition to functioning in hormone signaling in plant growth and development, SERKs complex with several RLP receptors to confer regulation in plant immunity (Albert et al., 2015; Fan et al., 2022; Heese et al., 2007; Zhang et al., 2014; Zhang et al., 2013).

The perception of PAMPs in PTI and activation of PRRs lead to several signaling outputs including phosphorylation of receptor-like cytoplasmic kinases (RLCKs), the influx of Ca^{2+} , production of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPK). FLS2 or EFR interacts with BAK1 upon flg22 or elf18 ligand perception to mediate downstream signaling. *Bak1* mutants show reduced ROS burst and MAPK activation upon elicitation, suggesting that BAK1 is a positive regulator in PRR immunity (Chinchilla et al., 2007). The identification of BIR1 (BAK1-interacting receptor like kinase) facilitated the identification of the LRR-RLK SOBIR1 (suppressor of BIR1), which constitutively associates with many RLPs like RLP42, RLP23 and RLP30 (Albert et al., 2015; Fan et al., 2022; Gao et al., 2009; Zhang et al., 2014; Zhang et al., 2013). The requirement of co-receptors is not merely established for LRR-type PRRs. A lysin motif receptor kinase LYK5, a major chitin receptor, interacts with a LysM receptor kinase CERK1 which is also involved in a complex with LYM1 LYM3 for sensing PGNs as a co-receptor (Willmann et al., 2011).

PRR-coreceptor complexes associate with BIK1 (BOTRYTIS-INDUCED KINASE 1), a member of the RLCK VII-8 subfamily, which is phosphorylated by BAK1 upon flg22 or elf18 treatment, indicating that BIK1 functions in PRR complex signaling (Lu et al., 2010). PBL1 (PBS1-Like1), the homolog of BIK1, interacts with FLS2 directly and is involved in immune signaling (Zhang et al., 2010). Unlike BIK1, which is phosphorylated by BAK1, PBL27 is phosphorylated by CERK1 in the chitin-induced immunity, indicating that PBL27 and BIK1 are involved in distinct PAMP signaling pathways (Shinya et al., 2014). PBL19 translocates

from the plasma membrane to the nucleus by chitin induction and phosphorylates EDS1, a central signal component of ETI, to activate immunity against fungi in Arabidopsis, while PBL30 and PBL31 are not essential for ETI^{RRS1-RPS4} and ETI^{RPS2}, but essential for PTI^{RLP23} function (Li et al., 2022; Pruitt et al., 2021; Rao et al., 2018). However, not all members in RLCK subfamily VII function positively in PTI, PBL13 is an exception that negatively regulates the MAPK3/6 activation and ROS burst elicited by flg22 in PTI^{FLS2} (Lin et al., 2015). In addition, several RLCKs act as central components in linking PRRs to trigger downstream defense (Liang & Zhou, 2018). Upon PAMP perception, BIK1 subsequently binds and phosphorylates NADPH oxidase RBOHD which plays a predominant role in ROS production and Ca²⁺-based regulation (Kadota et al., 2015). BSK1 (BR-SIGNALING KINASE1), a substrate of BIK1 in RLCK family, associates with FLS2 to regulate flg22-induced ROS burst positively (Shi et al., 2013). Furthermore, BSK5 is phosphorylated by EFR and PEPR1 and is required in PAMP/DAMP-induced ROS response which suggest that the RLCK XII subfamily plays an essential role in PTI (Majhi et al., 2019).

1.2.2 Early cellular signaling events

1.2.2.1 Ca²⁺ influx

A rapid increase of cytosolic Ca²⁺ influx from the apoplast triggered by PAMPs represents one early signaling event during PTI (Blume et al., 2000). The specific peak time, spatial and amplitude are different when treated with various PAMPs. Pep-13 generates a fast Ca²⁺ influx with peaking around 4 min after treatment, but flg22 needs around 2-3 min, while PGN activates much lower and increases for longer (Xu et al., 2022). However, Ca²⁺ elevation is much more continuous and sustainable in ETI than transient increases observed in PTI. DC3000 carrying AvrRpm1 effector elicits a sustained Ca²⁺ influx with 2 peaks around 150 min (Grant et al., 2000). Cyclic nucleotide-gated ion channels (CNGCs) CNGC2 and CNGC4 assemble in Arabidopsis and function as a calcium permeable channel upon flg22 treatment to maintain high external calcium concentration in the resting state. Once activated upon pathogen invasion, this channel is phosphorylated by BIK1, resulting in an increase of cytosolic calcium (Tian et al., 2019).

After application of the calcium-channel blocker lanthanum, the first Ca²⁺ peak is reduced and the expression of H₂O₂-induced gene *GST1* (Glutathione-S-Transferase 1) is moderated (Rentel & Knight, 2004). The Ca²⁺-dependent protein kinases (CPKs), which are Ca²⁺ sensors, decode and relay Ca²⁺ signals in the immune response upon activation of PRRs and NLRs via phosphorylating RBOHD, consisting of ROS and calcium signals which facilitate each other (Yuan et al., 2017).

1.2.2.2 ROS burst

ROS is produced by organelles with oxidizing activity during normal metabolism (Tripathy & Oelmuller, 2012), however, plants also produce ROS autonomously as signaling molecules to control biological processes and to defend against pathogens (Ishiga & Ichinose, 2016). ROS production was first reported to be a defense response upon treatment of the hyphal wall component (PAMP) from *Phytophthora infestans* in potato (Doke, 1983). As antimicrobial molecules protect against pathogen entry and secondary messengers trigger stomatal closure to limit pathogen growth at the first phase, ROS occurs at the second phase before cell death and cell collapse caused by necrotrophic pathogens (Shetty et al., 2003; Shetty et al., 2007). During plant-pathogen interaction, the NADPH oxidases, also called RBOHs, play a key role in the generation of ROS. In Arabidopsis RBOHD is associated with PRRs and is phosphorylated by BIK1, CPKs, and MAP4 kinase (SIK1) after PRR elicitation by PAMPs. The RBOHD phosphorylation sites S343 and S347 are crucial to BIK1-related PTI as well as RPS2-initiated ETI (Kadota et al., 2015; Kadota et al., 2014). RBOHD is also associated with RLCK PBL13 and the association is disrupted by flg22, and is phosphorylated by PBL13 at S862 and T912 sites for negative regulation (Lee et al., 2020; Lin et al., 2015). It was recently found that RIPK (RPM1-induced protein kinase), an RLCK VII subfamily member, regulates ROS production in plant immunity comprising of PTI, ETI, DTI (damage-associated molecular pattern-triggered immunity), and SAR (systemic acquired resistance) by trans-phosphorylating RBOHD at residues RBOHD S343/S347 (Li et al., 2021). In contrast to the rapid and transient ROS burst induced by PTI, ETI-ROS is produced in a biphasic manner with a much stronger and sustained second peak compared to the first (Chandra et al., 1996). The mechanism of ROS production is well known in PTI, however, is remaining uncovered in ETI. In recent study, *Nicotiana benthamiana* N protein (NLR) recognizes effector p50 derived from TMV and phosphorylates a transcriptional repressor NbAL7 which binds to ROS scavenging gene promoter to inhibit gene transcription for ROS accumulation (Zhang et al., 2023).

1.2.2.3 MAPK signaling

There are two MAPK cascades in plant immunity: the MAPKKK-MKK4/5-MPK3/MPK6 and MEKK1-MKK1/2-MPK4, pathways play an essential role in phosphorylation of downstream transcription factors (TFs), such as WRKY33 and other substrates like ACS isoforms to translate extracellular stimulation into intracellular responses. Of note, the connection between MAPK cascade activation and PRR complexes signaling is well-studied while elusive in ETI (Meng & Zhang, 2013; Yamada et al., 2016). Upon flg22, elf18, chitin, and pep2 treatment, phosphorylation of MAPKKK3/5 and MEKK1 by RLCK VII subfamily members are detected and required for pattern-triggered activation of MPK3/6 via MKK4-MPK5 and activation of MPK4 via MKK1/2, respectively (Bi et al., 2018). Different from the

strong and transient activation of MAPK cascades in PTI, the activation of MPK3/6 is much sustained upon effector AvrRpt2 treatment from *P. syringae* which is indirectly recognized by CNL RPS2 (Tsuda et al., 2013). Interestingly, AvrRps4-triggered MAPK activation, mediated by TNL proteins RPS4 and RRS1, only occurs once AvrRps4 is secreted by *Pseudomonas* or in presence of flg22. However, bacteria-delivered or transgenic AvrRpt2 enable MAPK activation independent of PRR signaling, indicating that in ETI the CNL- and TNL-triggered MAPK activation function differently (Ngou et al., 2020).

1.2.2.4 Plant hormone (Ethylene)

Ethylene (ET) is a gaseous phytohormone that is not only involved in the growth and development of plants, but also in biotic and abiotic stress regulation (van Loon et al., 2006). The process of ET synthesis is consisting of two steps: SAM (S-adenosylmethionine) is converted to ACC (1-aminocyclopropane-1-carboxylic acid) by ACS (ACC synthase); ACC is oxidized by ACO (ACC oxidase) to form C₂H₄ (ET) (S F Yang & Hoffman, 1984). ACS2 and ACS6 are the rate-limiting enzyme in ET synthesis and are phosphorylated by MPK6 which increases ACS activation and ET production (Liu & Zhang, 2004). WRKY33, a TF activated by MPK3/4/6, also regulates ACS2/6 gene expression by binding to their promoters (Li et al., 2012). During the immune response, ET accumulation is initiated upon PAMP treatment, such as 0.2 μM flg22 can trigger maximum ET production within 4 hours in 2-week-old Arabidopsis seedlings (Liu & Zhang, 2004). ROS burst is compromised in ET receptor mutants *etr1-1*, which demonstrates that ET signal is necessary in flg22-triggered ROS (Mersmann et al., 2010). As a result, the easy-to-detect system of ET response triggered by PAMPs has enabled the identification of ET-sensitive plants (containing receptors) and ET-insensitive (mutants or lack of receptors) by gas chromatography and has facilitated the discovery of new receptors and transformants (L. Wang et al., 2016). Except for ET, there are other phytohormones playing crucial roles as signal molecules in immunity like salicylic acid (SA) and jasmonate (JA).

1.2.2.5 Hypersensitive response (HR)

A rapid cell death which emerges at the site of pathogen penetration and infection is referred to as hypersensitive response (HR), which mediates disease suppression to limit early invasion (Balint-Kurti, 2019). HR is initially invoked by interaction of R genes and Avr proteins, mostly associated with NLRs and effectors, but is not commonly observed in PTI. As mentioned above, AvrRps4, an effector originally identified from *P. syringae*, is recognized by TNL RPS4/RPS1 pair and is able to trigger HR. Another effector from *P. syringae*, AvrRpt2 sensed by CNL RPS2 can also induce cell death while dependent on autophagy components, a different mechanism compared to AvrRps4. Like other responses, HR mediated by RPS2 and RPS4/RPS1 requires the participation of MPK3 and MPK6 (Su et al., 2018). ROS is an

important defense response for initiating HR. Although HR is an uncommon defense response in PTI, there are some RLPs, such Cf-4 and Cf-9 in *S. lycopersicum*, RLP42 in Arabidopsis, RXEG1 and REL in *N. benthamiana*, ELR and PERU in *S. tuberosum* that cause cell death upon PAMP infiltration (Chen et al., 2023; Du et al., 2015; Jones et al., 1994; Thomas et al., 1997; Torres Ascurra et al., 2023; Wang et al., 2018; Zhang et al., 2014).

1.3 A united plant immunity

Since the idea of plant immunity consisting of two branches, PTI and ETI, was presented in 2006 (zig-zag model), the mechanisms and outputs are separately researched until 2011. Then, a hypothesis was proposed that PTI and ETI may execute overlapping signaling elements (complex) for interaction and activation (Qi et al., 2011). In PTI, upon PAMPs elicitation, PRRs recruit BAK1 to form complex together with SOBIR1, phosphorylate RLCKs, activate immune response, and culminate in a basal and broad-spectrum resistance. In ETI, sensor NLRs and helper NLRs interact with effectors directly or indirectly to assemble resistosomes and transduce signaling via EDS1-PAD4-ADR1 or EDS1-SAG101-NRG1 for Ca²⁺ influx and cell death then finally provide robust response to inhibit pathogen proliferation. The fact that activation could not be studied in the absence of PTI has become hindrances for discovering the responses triggered by ETI alone.

Recent studies have revealed that PTI and ETI function mutually to prevent pathogen invasion. To eliminate the effects of PTI, the immunity conferred by conditional induction of AvrRps4 with oestradiol in Arabidopsis is exclusive to ETI but not PTI. After induction, ROS burst activated by flg22 is increased as well as co-treatment of flg22 and oestradiol enhances ROS production, especially in phase III, demonstrating that ETI^{AvrRps4} can enhance PTI immune outputs. Another paper provides evidence that the comprised ETI in SNIPER1 overexpression lines weakens PTI in the infection by *Pst* DC3000 *hrcC*, which indicates that activation of ETI can boost PTI (Ngou et al., 2021; Tian et al., 2021). The fact that ETI^{AvrRps2} is abolished in PRR/co-receptors mutants *fls2 efr cerk1* and *bak1 bkk1 cerk1* upon *Pst* DC3000 AvrRps2 infection shows that PTI is required for ETI (Yuan et al., 2021). In TNL signaling mutants *eds1 pad4 adr1-triple* but not *sag101 nrg1-triple*, expression of plant immunity genes and SA level induced by nlp20 are decreased, echoing the ethylene production elicited by nlp20 is impaired in *eds1, pad4, adr1* but not in *sag101* and *nrg1* (Pruitt et al., 2021; Tian et al., 2021). These two findings collectively delineate that ETI signaling components are engaged in PTI. Together, PTI and ETI are both required for and potentiate mutually immunity to mount robust resistance to infection (Chang et al., 2022).

1.4 Plant pathogens

Plants are threatened by pathogenic microorganisms present throughout their growth, including bacteria, fungi, oomycetes, and viruses. In the lifecycle of pathogen infection, pathogens undergo adhesion of plant surfaces, entrance of tissues, colonization, reproduction, and dissemination to other tissues or new hosts. Every stage of infection is immersed in plant defense challenges. By means of research of the interaction between different plant pathogens and model plants under laboratory systems, pathogenic and host-resistant mechanisms are revealed, which are crucial for controlling impact of plant disease on agriculture.

The bacterial, fungal, and oomycete species described below are chosen for representing each kingdom and are associated with the work in this thesis.

1.4.1 Bacteria

Pseudomonas syringae, as a superior model plant pathogenic bacterium, is a Gram-negative phytopathogenic bacterium that can cause plant disease on most plant tissues and economic losses on crops including tomato and tobacco (Rooney et al., 2020). As a hemibiotrophic pathogen, *P. syringae* initially stays on plant surface, then invades the apoplast through wounds and stomata, secretes phytotoxins and effectors to overcome plant immunity, and causes water soaking, chlorotic and eventually necrotic symptoms (Chakravarthy et al., 2018). Currently, approximately 64 pathovars are determined based on host plant species and disease phenotype (Bundalovic-Torma et al., 2022). *P. syringae* pv. *tomato* (*Pst*) DC3000 strain is extensively used for the study of plant-bacterial interaction mechanisms in *Arabidopsis* (Katagiri et al., 2002; Xin & He, 2013).

To initiate plant infection via stomata, *P. syringae* has evolved phytotoxins and effectors to conquer this first barrier (Xin et al., 2018). Plants detect PAMPs derived from *P. syringae* like flagellin through PRRs in PTI, which triggers stomatal closure to prevent further bacterial entry. *P. syringae* uses the phytotoxin coronatine, which simulates the plant hormone jasmonate, to activate stomatal opening, and compete with salicylic acid signaling (Zheng et al., 2012). Bacteria also employ the type III secretion system (T3SS) to inject effectors to trigger jasmonate signaling and inhibit ROS and stomatal closure induced by PTI. In this process, high humidity and cool temperature facilitate the population, movement, and survival time of *P. syringae* (Yao et al., 2023).

After entering the apoplast, *P. syringae* faces the defense elicited not only by PTI but also ETI. T3SS effectors are ready to suppress plant immunity, for example, AvrPtoB inhibits or degrades PRRs like FLS2, CERK1, and BAK1 by E3 ubiquitin ligase activity (Gimenez-Ibanez et al., 2009). However, AvrRpt2 cleaves RIN4 in the plant cell, resulting in the

degradation of RIN4 and disruption of the interaction between RIN4 and RPM1 which activates the HR subsequently (Mackey et al., 2003). As PTI and ETI are perturbed by effectors, plant immunity outputs like ROS, callose deposition, and HR are somehow suppressed. HopM1 and AvrE promote water soaking by targeting MIN7 and PP2A, respectively, which are involved in host vesicle trafficking (Xin et al., 2016). Pathogen proliferation in the apoplast and disease outbreak are the consequences of a concerted action including a virulent pathogen, a susceptible host, and an appropriate environment.

1.4.2 Fungi

Botrytis cinerea is a widespread necrotrophic plant fungus that can infect more than 200 plant species and that causes gray mold disease in many economically important vegetables and fruits like tomato, berries, and grapes. Unlike *Pseudomonas* which can deliver effectors into plant cells, *Botrytis* produces toxins, enzymes, and other compounds to degrade and kill host cells. The life cycle of *Botrytis* includes infection of conidia, penetration, invasion on infected tissue, and formation of new conidia (AbuQamar et al., 2017; Williamson et al., 2007).

Conidial infection is initiated upon production of conidia by sclerotia which is affected by temperature, humidity, and UV. High humidity and cool temperature (10-16°C) facilitate the generation of conidia, while dry conditions or water droplets can shed conidia into air or adjacent plant tissues. As UV can stimulate the formation of conidia, it is exploited for conidia generation under laboratory conditions (Bi et al., 2023).

When conidia attach to plant tissues and germinate under the right conditions, a great battle is imminent. *Botrytis* enters the host through stomata and wounds or directly penetrates the cuticle with conidial germ tubes which can form appressoria or infection cushions for assistance of infection. To establish and develop infection in host cells, *Botrytis* secretes toxins such as botcinic acid and botrydial, and cell death inducing-proteins to kill host cells. It also secretes cell wall-degrading enzymes to break down plant cell wall in order to facilitate fungal growth and acquisition of nutrients. *Bcpg1*, which encodes a endopolygalacturonase, plays an indispensable role in fungal virulence (Have et al., 1998). With the exception of these compounds produced by *Botrytis*, the fungus manipulates its own ROS production and induces plant ROS to promote cell death. Deletion of *BcnoxA* and *BcnoxB* which encode two NADPH oxidases results in no sclerotia formation and impaired pathogenicity (Segmüller et al., 2008). To cope with infection, plants produce antimicrobial compounds, strengthen cell walls, and utilize transcription factors to activate plant defense. Transcription factor families involving ERFs, MYBs, NACs, MYCs, and WRKYs serve as signaling components in plant defense (Liu et al., 2017). In *Arabidopsis*, the expression of BIK1 (Botrytis induced kinase1)

is induced by *Botrytis* infection which demonstrates the link between PTI and *Botrytis* infection.

Accumulation of fungal biomass, spread of lesions, colonization, and cell death until tissue decomposition eventually lead to the formation of sclerotia and the new generation of conidia.

1.4.3 Oomycetes

Albugo laibachii is an obligate biotrophic oomycete which generally exists in Arabidopsis in the natural environment, while *Phytophthora capsici* and *Phytophthora infestans* are hemibiotrophic microorganisms that are not capable of threatening Arabidopsis in the wild, but are destructive to crops and fruits, especially *P. infestans* is the cause of notorious potato late blight (Herlihy et al., 2019). Due to different lifestyles, disease symptoms range from white blisters and rot to water-soaked chlorotic spots, which have a significant impact on plants.

Through water and wind, the asexual spores released by the sporangium are dispersed and attach to plant tissue (Judelson & Ah-Fong, 2019). After germination, the germ tubes of *Albugo* enter the hosts through penetration of appressoria or via stomata or epidermis and form a specialized structure, the haustoria, which can absorb nutrients from the host to feed on itself and secrete effectors and hydrolases into plants to suppress plant immunity. Along with the growth of hyphae in cells developed into mycelia, the intracellular spaces are occupied and infected until the hyphae extend from the stomata to form a sporangiophore which can directly germinate a sporangium, and the asexual cycle is completed (Grenville-Briggs & van West, 2005). The sexual reproduction of *P. capsici* is utilized to survive in soil since the thick-walled oospores overcome adverse conditions and infect roots once the oospores attach to plant tissues (Quesada-Ocampo et al., 2023). As hemibiotrophic pathogens, instead of being compatible with these hosts, *P. capsici* and *P. infestans* trigger necrosis until cell death after 2-3 days (Sanogo et al., 2023).

Since few oomycetes successfully infect Arabidopsis under natural and experimental conditions, *Albugo* is not only a feasible pathosystem to investigate the interaction between Arabidopsis and oomycetes, but also facilitates the infection of incompatible oomycete, *P. infestans*, which provides additional resources for further study (Belhaj et al., 2017). To detect oomycetes and activate defense, plants employ receptors such as RLP23, RXEG1, and ELR to recognize the corresponding PAMPs nlp20, XEG1, and elicitors.

1.5 Strategies for achieving broad-spectrum resistance in plant breeding

Food is vital for all animals and humans. The continuous growth of the global population and climate change have accelerated the demand for food production and to minimize field losses. Plant diseases have a significant impact on the crop yields and economic losses,

resulting in 20%-40% loss of food crops like wheat, rice, potato and maize (Oerke & Dehne, 2004). The most infamous example is *P. infestans*, the potato late blight, which resulted in the Irish potato famine that took over 2 million lives. To cope with and mitigate the consequences of plant disease outbreaks, integrated management mainly includes the application of conventional methods such as chemical pesticide control and crop management, and non-chemical protection such as the deployment of resistant genetic cultivars. Since synthetic pesticides are environmentally unfriendly and cost-prohibitive, host genetic resistance is critical for modern crop protection to achieve sustainable agricultural development and low-input but efficient control (Ristaino et al., 2021).

Host genetic resistance is generally divided into two types: race-specific and non-race-specific resistance. Race-specific resistance, also known as R-gene-mediated resistance, is commonly conferred by a major R gene. By the identification of full arsenal R genes from plants with innate resistance, potential resources for introgression are obtained (Poland & Rutkoski, 2016). For example, Rpi-blb1 originates from the wild potato *Solanum bulbocastanum* and confers resistance to *P. infestans* in the susceptible cultivar *S. tuberosum* (van der Vossen et al., 2003). However, due to the sexual reproduction of plant pathogens, high mutation rates in pathogens enable individual races to escape immunity resulting in loss of resistance (Richard et al., 2022).

Unlike race-specific resistance with limited durability, non-specific resistance is more durable which is a consequence of the recognition mechanism rarely affected by pathogen selection. To achieve durable and broad-spectrum resistance, there are several molecular breeding strategies.

1.5.1 Quantitative trait loci (QTL) and QTL combining with R genes

QTLs encode typical PRRs, atypical NLRs, signaling components, transporters, and enzymes to confer resistance in crops. QTLs are extensively applied in crops, especially in wheat, for instance, Lr67/Yr46 genes in wheat accession RL6077 act as resistance genes to stem rust and powdery mildew that can be introgressed into recombinant inbred lines (Herrera-Foessel et al., 2014). While QTL is not as sufficient to defend against pathogens as R genes and a single R gene is not capable of providing durable resistance, so the combination of QTL and R genes is effective and durable against one or several pathogen species. In wheat, the combination of Sr2, Lr34 or Lr68 increases resistance to leaf rust compared to a single Sr2 (Silva et al., 2015).

1.5.2 PRR-transfer

Since PAMPs are conserved among diverse isolates in one or several pathogens, interfamily transfer of PRRs is likely to confer durable resistance to one or multiple pathogen species. A successful example is that the Arabidopsis EFR transferred into crop plants, tomato, potato,

tobacco, wheat, and rice, not only conferred perception of elf18 but also improved resistance to bacteria (Boschi et al., 2017; Lacombe et al., 2010; Schoonbeek et al., 2015; Schwessinger et al., 2015; Zipfel et al., 2006). With various PRRs identified and transferred into crops recently, RLP23-expressing potato was resistant to bacteria, fungi, and oomycete, owing to the wide-spread NLP in diverse pathogens (Albert et al., 2015). Thus, the arsenal PRRs can be used for genetic engineering resistance between plant species, and chimeric receptors combined with PRRs can expand detection amplitude.

1.5.3 Receptor structure modification

As mentioned PRRs function distinctly from R proteins, with ligand binding and recognition occurring in the ectodomains and recruitment and signaling transduction in the intracellular domain. Since ligand binding capabilities are often limited to certain plant species or genera or families, and since intracellular signaling pathway are rather confused among plant species, the interfamily transfer of receptors is a suitable way to confer resistance to other species. Therefore, the generation of chimera receptors is critical to enhance crop immunity and improve transformation efficiency, such as the chimeric receptor EFR::XA21 confers recognition of elf18 and immune response in rice, demonstrating that the engineering and transformation of chimeric receptor are feasible for crop resistance and break the recognition limitation among species (Schwessinger et al., 2015).

1.5.4 Resistance gene stacking

Since interfamily transfer of resistance genes like PRRs, NLRs are impressive to enhance resistance among species, such as the classic Arabidopsis EFR receptor confers resistance in Solanaceous plants to bacteria, barley MLA1 (NLR) activates immunity to barley powdery mildew in Arabidopsis, the interfamily transfer approach is suitable and improvable for engineering immunity in crops.

To achieve more effective and economical breeding strategies, it has been proposed that pyramid resistance genes defend against multiple pathogens and/or achieve more immunity to single pathogen species. The classic approach for gene-stacking in breeding is crossing single overexpression lines to engineer plants. Arabidopsis EFR and pepper BS2 can confer resistance to bacteria *Ralstonia solanacearum* and *Xanthomonas perforans* in tomato, respectively. After crossing, the tomato with EFR/BS2 combination can reduce the growth of these two bacteria (Kunwar et al., 2018). Since drought stress genes indeed improve plants drought tolerance but affect growth, it is pragmatic to stack abiotic-stress-enhancing DREB1A with growth-enhancing PIF4 in Arabidopsis by crossing single transformants (Kudo et al., 2019). However, crossbreeding is time-consuming and strenuous, especially in crops. As the transgenesis technology in crops is constantly developing and maturing, the generation of gene-stacking in breeding is achieved by introducing a transgene cassette composed of

multiple genes into crops instead of crossing. Through advanced cloning technologies such as Gateway recombination strategy, the transgenic cassette containing four NLR genes and one hexose transporter gene on an expression vector was constructed and transferred into wheat by *Agrobacterium*-mediated transformation, which not only demonstrates the feasibility of gene-stacking but also overcomes short-term resistance caused by effector mutations (Luo et al., 2021). In addition to durable defense, gene stacking has been used to engineer broad-spectrum resistance by pyramiding three R genes *Rpi-vent1.1*, *Rpi-stol* and *Rpi-blb3* from wild potato into the cultivars Désirée and Victoria, conferring complete resistance (Ghislain et al., 2019). The stacking of PRRs, PERU and RLP23, conferred potato the perception of Pep-13 and nlp20 and resistance to *P. infestans* (Ascurra et al., 2023). Thus, the stacking of multiple genes constitutes a promising strategy to obtain durable and/or broad-spectrum resistance in breeding.

There are other approaches as well for plant breeding strategies such as inducible expression of *Avr* genes, host-susceptible gene editing, and the expression of components of plant defense downstream signaling (Li et al., 2020).

1.6 Aim of this study

A variety of PRRs were discovered in plants and interfamily transfer of single PRR was shown to confer resistance to crops. To investigate whether stacking (or pyramiding) of PRRs that recognizing multiple PAMPs derived from distinct microbial species or one pathogen species can confer a broad-spectrum or robust resistance to infection, the PRRs SICORE, SIEIX2, SLFLS3, and SmELR from crops were selected to be stacked and stably transformed into *A. thaliana* and *N. benthamiana* model plants. These plants possess shorter regeneration times than crops. In parallel, PRRs from Arabidopsis, including EFR, RLP23, and RLP42 were stably transformed into tomato and potato, and their contribution to immune response was investigated. On the other hand, higher-order mutants lacking multiple PRRs in Arabidopsis were generated and their susceptibility to pathogens was studied.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Chemicals applied to this thesis were purchased from the following companies: Carl Roth (Karlsruhe), Sigma-Aldrich (Taufkirchen), Duchefa (Haarlem, Netherlands), Merck (Darmstadt), Invitrogen (Karlsruhe). Restriction enzymes, ligases and DNA assembly and cloning kits were purchased from Thermo Fisher Scientific (St. Leon-Rot) and New England Biolabs (Frankfurt am Main). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg). Kits for PCR product purification and plasmid isolation were purchased from Thermo Fisher Scientific (St. Leon-Rot).

The peptides were synthesized from Genscript Inc. (Rijswijk, Netherlands). Crude peptides csp22, flg28, flg22, nlp20, and pg13 were dissolved in DMSO and kept at -20°C as 10 mM stock solution. Xylanase from *Trichoderma viride* was purchased from Sigma Aldrich and dissolved in DMSO and kept at -20°C (250 units was regarded as 1 mg, dissolved in 1 ml DMSO and the concentration was 45 µM).

The α-GFP primary antibody was purchased from Torrey Pines Biolabs, α-Myc and α-HA were from Sigma Aldrich. The second antibodies α-rabbit-IgG HRP, α-mouse-IgG HRP were ordered from Sigma Aldrich. ECL Detection Reagents and Nitrocellulose Membranes were ordered from Cytiva.

2.1.2 Media and antibiotics

Table 2.1. Medium used for vector construction, pathogen cultivation and plant transformation

Medium	Ingredients (for 1L)	Uses
LB	10 g Bacto-Trypton, 5 g Bacto-Yeast extract, 5 g NaCl, 15 g Agar	Cultivation of <i>E. coli</i> , <i>Agrobacterium</i>
YEB medium	5 g Beef-Extract, 1 g Yeast-Extract, 5 g Peptone, 5 g Sucrose, 0.49 g MgSO ₄ • 7H ₂ O, 15 g Agar	Cultivation of <i>Agrobacterium</i>
½ MS	2.2 g/l MS (Duchefa), 20 g Sucrose, pH to 5.7 with KOH. 8 g Select-agar.	Selection of Arabidopsis transformants
MS2%	4.31 g MS (Duchefa), 20 g Sucrose, pH to 5.7 with KOH, 7 g Daishin Agar	Stable transformation of <i>N. benthamiana</i>
Selection medium	4.31 g MS (Duchefa), 16 g Glucose, pH to 5.7 with KOH, 7 g Daishin Agar, after autoclaving, cool to 60°C, add 1 mg BAP, 0.2 mg NAA, 500 mg Cefotaxime and corresponding antibiotics.	
Rooting medium	4.31 g MS (Duchefa), 20 g Sucrose, pH to 5.7 with KOH, 7 g Daishin Agar, after autoclaving, cool to 60°C, add 500 mg Cefotaxime and corresponding antibiotics.	
Callus induction	4.31 g MS (Duchefa), 16 g Glucose, pH to 5.7 with KOH, 7 g Daishin Agar, after autoclaving, cool to 60°C, add 0.1	Stable transformation of potato

Material and Methods

medium	mg BAP, 5 mg NAA, 250 mg TiCl ₄ .		
Selection medium	4.31 g MS (Duchefa), 16 g Glucose, pH to 5.7 with KOH, 7 g Daishin Agar, after autoclaving, cool to 60°C, add 1.4 mg Zeatin riboside, 20 µg GA ₃ , 250 mg TiCl ₄ and corresponding antibiotics.		
Rooting medium	4.31 g MS (Duchefa), 16 g Glucose, pH to 5.7 with KOH, 7 g Daishin Agar, after autoclaving, cool to 60°C, add 250 mg TiCl ₄ and corresponding antibiotics.		
Germination Medium (GM)	4.31 g MS (Duchefa), 30 g Sucrose, pH to 5.7 with KOH, 7 g Daishin Agar.	Stable transformation of tomato	
Liquid Germination Medium	GM medium without agar.		
Conditioning Medium	GM medium add 0.1 mg BAP and 1 mg NAA		
Selection Medium	GM medium add 1 mg trans-Zeatin, 250 mg TiCl ₄ , and corresponding antibiotics.		
Rooting Medium	GM medium add 0.1 mg IAA, 500 mg Vancomycin, and corresponding antibiotics.		
King' B Medium	20 g Glycerol, 40 g Proteose Pepton Nr.3, 15 g Agar, after autoclaving, add sterilized 10 ml 10% K ₂ HPO ₄ , 10 ml 10% MgSO ₄ (or order from Carl Roth)		Cultivation of <i>P. syringae</i>
PDA	200 g Potato infusion, 20 g Glucose, 20 g Agar (or order from Carl Roth)		Cultivation of fungi and oomycete
PDB	200 g Potato infusion, 20 g Glucose (or order from Carl Roth)		
MEA	30 g Malt extract, 5 g Mycological peptone, pH to 5.4, 15 g Agar (or order from Thermo Fisher Scientific)	Sporulation of <i>B. cinerea</i>	
10% V8 juice	100 ml V8 juice (from Amazon), 1 g CaCO ₃ , 15 g Agar	Sporulation of <i>P. capsici</i>	
RS	60 g bio-rye soak in 200 ml H ₂ O for 2 days, chop in blender, incubate at 50°C water bath for 3 h, filtrate supernatant, 20 g Sucrose, pH to 7 with NaOH, 15 g Agar, after autoclaving, cool to 60°C, add 50 µg/ml carbenicillin and 10 µg/ml rifampicin against bacteria.	Sporulation of <i>P. infestans</i>	

Table 2.2. Antibiotics

Antibiotic and hormones	Final concentration(µg/ml)	Solvents
Rifampicin	50	DMSO
Kanamycin	50	H ₂ O
Gentamycin	40	H ₂ O
Spectinomycin	75	H ₂ O
Cefotaxime	250	H ₂ O
Carbenicillin	50	H ₂ O
Basta	4	H ₂ O
Ticarcillindisodium	250	H ₂ O
Vancomycin	500	H ₂ O
trans-Zeatin	1	1/10 0.1 M HCl and 9/10 H ₂ O
α-Naphthaleneacetic acid	1	1/10 0.1 M NaOH and 9/10

(NAA)		H ₂ O
6-BenzyAminoPurin (BAP)	0.1	1/10 0.1 M NaOH and 9/10 H ₂ O
Indole-3-Acetic Acid (IAA)	0.1	1/10 100% EtOH and 9/10 H ₂ O
Gibberellic acid (GA3)	0.02	H ₂ O
Zeatin riboside (ZR)	1.4	1/10 0.1 M HCl and 9/10 H ₂ O

Table 2.3. Plasmid used in this thesis

Constructs	Features	Origins	
pCR8/GW/TOPO	pUC origin, rrnB T1 and T2, attL1, attL2, Spec	Nürnberg lab	
pENTR/D-TOPO	pUC origin, rrnB T1 and T2, attL1, attL2, Kanr		
pGWB14	35S promoter, C-3×HA, attR1 ccdB attR2, Kanr (bacteria and plant)		
pGWB20	35S promoter, C-10×Myc, attR1 ccdB attR2, Kanr (bacteria and plant)		
pB7FWG2.0	35S promoter, C-eGFP, attR1 ccdB attR2, Spec (bacteria), Basta (plant)		
pK7FWG2.0	35S promoter, C-eGFP, attR1 ccdB attR2, Spec (bacteria), Kanr (plant)		
pB7FWG2.0_SICORE	35S promoter, C-eGFP, attR1 SICORE attR2, Spec (bacteria), Basta (plant)	This work	
pB7FWG2.0_SIEIX2	35S promoter, C-eGFP, attR1 SIEIX2 attR2, Spec (bacteria), Basta (plant)		
pGWB14_SIEIX2	35S promoter, C-3×HA, attR1 SIEIX2 attR2, Kanr (bacteria and plant)		
pGWB20_SIEIX2	35S promoter, C-10×Myc, attR1 SIEIX2 attR2, Kanr (bacteria and plant)		
pGWB20_SIFLS3	35S promoter, C-10×Myc, attR1 SIFLS3 attR2, Kanr (bacteria and plant)		
pGWB20_SmELR	35S promoter, C-10×Myc, attR1 SmELR attR2, Kanr (bacteria and plant)		
pB7FWG2.0_SICORE-SmELR	35S promoter, C-eGFP, attR1 SICORE attR2; 35S promoter, C-10×Myc, attR1 SmELR attR2, Spec (bacteria), Basta (plant)		
pB7FWG2.0_SICORE-SIFLS3	35S promoter, C-eGFP, attR1 SICORE attR2; 35S promoter, C-10×Myc, attR1 SIFLS3 attR2, Spec (bacteria), Basta (plant)		
pB7FWG2.0_SICORE-SmELR-SIEIX2	35S promoter, C-eGFP, attR1 SICORE attR2; 35S promoter, C-10×Myc, attR1 SmELR attR2; Ubq10 promoter, C-3×HA, attR1 SIEIX2 attR2, Spec (bacteria), Basta (plant)		
pB7FWG2.0_SICORE-SIFLS3-SIEIX2	35S promoter, C-eGFP, attR1 SICORE attR2; 35S promoter, C-10×Myc, attR1 SIFLS3 attR2; Ubq10 promoter, C-3×HA, attR1 SIEIX2 attR2, Spec (bacteria), Basta (plant)		
pGWB5_StPERU	35S promoter, C-eGFP, attR1 StPERU attR2, Kanr (bacteria and plant)		
pK7FWG2.0_AtEFR	35S promoter, C-eGFP, attR1 AtEFR attR2, Spec (bacteria), Kanr (plant)		
pGWB14_AtRLP23	35S promoter, C-3×HA, attR1 AtRLP23 attR2, Kanr (bacteria), Kanr (plant)		Dr. Isabell Albert

pLOC _G _AtRLP42	35S promoter, C-eGFP, attR1 AtRLP42 attR2, Kanr (bacteria), Kanr (plant)	Dr. Lisha Zhang
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Table 2.4. Primers used in this thesis

Oligo Name	Sequence (5'-3')	Characteristics
SICORE_FW	ATGGTGAAAGGGAATGAAACAGAC	pCR8/TOPO cloning, sequencing
SICORE_REV	TAACTTTTTCTTCCGGTATGCTTG	
SIEIX2_FW	CACCATGGGCAAAAGAACTAATCCAAGAC	pENTR/TOPO cloning, sequencing
SIEIX2_REV	GTTCCCTTAGCTTTCCCTTCAGTC	
SmELR_FW	ATGGTCATGAGTCTGTTTTCTTT	pCR8/TOPO cloning, sequencing
SmELR_REV	AGTCCTTCGTCTCTGAGCTCTC	
SIFLS3_FW	ATGCTTAGTA ACATCATGGA GAAAC	pCR8/TOPO cloning, sequencing
SIFLS3_REV	ATTTACTTCTATGTTTCCAAATGTG	
SICORE_FW1	ATGGACTCACAGGAAAAGTACC	SICORE sequencing and genotyping
SICORE_REV2	TGATTTCTTTCTTTCTTTACTCC	
SIFLS3_REV1	TGCCTAATGAAGCAGGAATTGTTG	SIFLS3 sequencing and genotyping
SmELR_FW1	TTGACAAATCTGGAGCTACTTTCTc	SmELR sequencing and genotyping
SmELR_REV2	AAGGTTTAGGAACTGCAACGAGTC	
SIEIX2_REV	CCTCCAATATCCAAGATTTGCAG	SIEIX2 sequencing and genotyping
P35S_FW	GAAGTTCATTTCAATTTGGAGAGAAC	35S promoter sequencing
GB_Apa1_FW	GACCTGCAGGCATGCGACGTCGGGCCA GATTAGCCTTTTCAATTTCAAGAAAGTGC CTAACCC	Amplification of SmELR or SIFLS3 combined to pB7FWG2.0_SICORE for Gibson Assembly
GB_Apa1_REV	GGTACCCGGGGATCCTCTAGAGGGCCG ATCTAGTAACATAGATGACACCGC	
GB_UBQ10_FW	GGTTTACCCGCCAATATATCCTGTCAAAC ACTGATAGTTTGACGAGTCAGTAATAAAC GGCGTCA	Amplification of UBQ10 combined to pB7FWG2.0_SICORE-SmELR or pB7FWG2.0_SICORE-SIFLS3 for Gibson Assembly
GB_UBQ10_REV	GTCTTGGATTAGTTCTTTTGCCATTGTT AATCAGAAAACTCAGATTAATCTAC	
GB_EIX2_FW	CTTGAGCTTGGATCAGATTGTCGTTTCCC GCCTTCAGTTTGATCTAGTAACATAGATG ACACCGCGC	Amplification of SIEIX2 combined to pB7FWG2.0_SICORE-SmELR or pB7FWG2.0_SICORE-SIFLS3 for Gibson Assembly
GB_EIX2_REV	TTAATCTGAGTTTTTCTGATTAACAATGG GCAAAGAATAATCCAAGAC	
AtCERK1_FW	ATGAAGCTAAAGATTTCTCTAATCGCTCC G	Quintuple mutants <i>efr fls2 cerk1 lym3 sobir1</i> genotyping
AtCERK1_REV	CTACCGCCGGACATAAGACTGAC	
AtLyM3_FW	ATGAAGAATCCAGAAAAACCCTTACTTC	
AtLyM3_REV	TTAGAAAACAAAAAGCAAGAACCAATGG	
AtRLP23_FW	ATGTCAAAGGCGCTTTTGCATTTGCAT	RLP23 genotyping
AtRLP23_REV	GGTAACGTAGCTGGTGCAACTC	
AtRLP42-FW	ATGTCTAAATCGCTTTTGCCTTTGAC	RLP42 genotyping
AtRLP42_REV	CAAGACCTCTAGTTTGTGAGATTGC	

2.2 Organism

2.2.1 Bacteria

Escherichia coli

E. coli strain DH5 α was grown in LB medium with corresponding antibiotics at 37°C overnight.

Agrobacterium tumefaciens GV3101

The GV3101 strain with rifampicin and gentamicin resistance was grown on LB or YEB medium with corresponding antibiotics at 28°C for 2 days, in liquid culture for 24 h.

Pseudomonas syringae

The *P. syringae* pv. *tomato* (*Pst*) DC3000 and *P. syringae* pv. *tabaci* (*Pstab*) strains were grown on the King's B medium with rifampicin at 28°C for 2 days, in liquid culture for 24 h.

2.2.2 Fungi

Botrytis cinerea

B. cinerea strain B05.10 was grown on the PDA medium at 20°C for 5 days until the hyphae covered the whole plate.

Sporulation

B. cinerea B05.10 was grown on the MEA medium in the dark at 20°C, after 3-4 days, the plate was placed under near-UV light (350-400 nm) in the biosafety cabinet to promote sporulation overnight and relocated to darkness for 4-5 days. The spores were washed with 10-20 ml H₂O and the suspension was filtered by a cell sieve (EASYstrainer 40 μ M). The filtrate was centrifuged at 1200 rpm for 5 min and the supernatant was removed. Spores were precipitated and resuspended with quantitative H₂O.

The spore suspension was counted under a microscope by hemocytometer to desired density and stored in a -80°C freezer with 70% glycerin in the concentration of 1x10⁷ spores/ml.

2.2.3 Oomycete

Phytophthora capsici

Agar plugs of *P. capsici* previously cultivated on PDA plate were incubated on V8 plate for 3-4 days. Subsequently, the mycelium was cut into 15 pieces in 3 mm² and put into 25 ml 1/10 V8 juice liquid in a petri dish. The petri dish was incubated on the bench top at RT and shaken gently to prevent hyphae from sticking.

Material and Methods

The day before inducing zoospores, the V8 liquid was poured out and 10-20 ml cold H₂O was added. The petri dish was kept at 4°C for 1 h, and placed on the bench top at RT for 30 min.

The concentration of zoospores was counted by hemocytometer under a microscope and adjusted to 1000-2000 zoospores/ml.

Phytophthora infestans

Agar plugs of *P. infestans* were inoculated on RS plate for 3-4 days to activate hyphae. The plate was added with 5-10 ml H₂O to cover all hyphae, and a coating rod was used to remove the air. The plate was placed at 4°C for 1-2 h and restored at RT for 30 min. The suspension was filtrated and spinned down at 1500 rpm for 2 min. The zoospores were observed by microscope and counted by hemocytometer.

Albugo laibachii

The obligately biotrophic parasite *A. laibachii* race Nc14 was propagated on infected Arabidopsis Col-Tho and preserved in a Phyto incubator. Zoosporangia from 14-day-infected plants were collected by suspending the leaves with white blisters in cold water on ice for 1 h. The suspension was filtrated by a cell sieve (EASYstrainer 40 µM) and zoosporangia were counted by hemocytometer under the microscope.

2.2.4 Plants

Arabidopsis thaliana

Wild-type: Col-0

Mutant lines: *efr fls2*, *efr fls2 sobir1*, *efr fls2 cerk1*, *efr fls2 cerk1 lym3* (Col-0 background)

For experiments, the plants were grown on the potting soil with Gnatrol SC in the Phyto chamber under short-day conditions (22°C, 8 h 150 µmol/cm²s light, 40-60% humidity) and used at an age of 5-6 weeks.

For collecting seeds, the plants were grown on the potting soil in the greenhouse.

Table 2.5. *A. thaliana* transgenic lines used in this thesis

Name	Construct of transformation	Origin
CORE # 3, # 12	pB7FWG2.0::p35S-SICORE-GFP	This work
ELR # 1, # 3	pGWB20::p35S-SmELR-Myc	
EIX2 # 2, # 6	pB7FWG2.0::p35S-SIEIX2-GFP	
CORE-ELR # 3, # 9	pB7FWG2.0::p35S-SICORE-GFP-p35S-SmELR-Myc	
CORE-ELR-EIX2 # 13, # 14	pB7FWG2.0::p35S-SICORE-GFP-p35S-SmELR-Myc-Ubq10-SIEIX2-HA	

Nicotiana benthamiana

For experiments, plants were grown in the greenhouse and used at an age of 4 weeks under long-day conditions (22°C, 16 h 150 $\mu\text{mol}/\text{cm}^2\text{s}$ light, 40-60% humidity).

Table 2.6. *N. benthamiana* transgenic lines used in this thesis

Name	Construct of transformation	Origin
CORE # 1, # 5	pB7FWG2.0::p35S-SICORE-GFP	This work
FLS3 # 1, # 5	pGWB20::p35S-SIFLS3-Myc	
EIX2 # 4, # 10	pGWB20::p35S-SIEIX2-Myc	
CORE-FLS3 # 9, # 13	pB7FWG2.0::p35S-SICORE-GFP-p35S-SIFLS3-Myc	
CORE-FLS3-EIX2 # 1, # 3	pB7FWG2.0::p35S-SICORE-GFP-p35S-SIFLS3-Myc-Ubq10-SIEIX2-HA	

Solanum lycopersicum and *Solanum tuberosum*

The *Solanum lycopersicum* m82 and *Solanum tuberosum* Désirée plants were grown in the greenhouse for experiments and for collecting tomato seeds under long-day conditions (22°C, 16 h 150 $\mu\text{mol}/\text{cm}^2\text{s}$ light, 40-60% humidity).

2.3 Methods

2.3.1 Molecular biology methods

2.3.1.1 Plasmid DNA extraction

The extraction of plasmid DNA was carried out by the GenJET Plasmid Miniprep Kit, the concentration was measured by NanoDrop 2000 spectrophotometer at 220-340 nm.

2.3.1.2 Polymerase Chain Reaction (PCR)

The standard PCR reaction was set up with DreamTaq DNA polymerase and Phusion High-Fidelity DNA polymerase (Thermo Scientific). For cloning, the Phusion High-Fidelity DNA polymerase was performed accurately and quickly. The DreamTaq DNA polymerase was used to confirm the transformants and all standard PCR applications. The PCR protocol and cycling instructions were followed with tables respectively.

Table 2.7. PCR for cloning

Phusion High-Fidelity DNA polymerase reaction mix

Component	50 μl
ddH ₂ O	32.5 μl
5 × Phusion HF buffer	10 μl
10 mM dNTP	1 μl
10 μM Primer F	2.5 μl
10 μM Primer R	2.5 μl
polymerase	0.5 μl
Template DNA (1 pg-10 ng)	1 μl

Material and Methods

Cycling instruction

Initial Denaturation	98°C	30 s	25-35 cycles
Denaturation	98°C	10 s	
Annealing	Melting temperature	30 s	
Extension	72°C	30 s/kb	
Final Extension	72°C	10 min	

DreamTaq DNA Polymerase reaction mix

Component	50 µl
ddH ₂ O	37.7 µl
10 × DreamTaq buffer	10 µl
2 mM dNTP	5 µl
10 µM Primer F	0.5 µl
10 µM Primer R	0.5 µl
polymerase	0.3 µl
Template DNA (10 pg-1 µg)	1 µl

Cycling instruction

Initial Denaturation	95°C	3 min	25-40 cycles
Denaturation	95°C	30 s	
Annealing	TM	30 s	
Extension	72°C	1 min/kb	
Final Extension	72°C	10 min	

2.3.1.3 Cloning

TOPO cloning

The PCR products were checked by agarose gel electrophoresis and purified by the GeneJET PCR Purification Kit, and subsequently cloned into the desired vectors.

Table 2.8. TOPO cloning reaction

Reagent	Volume
PCR product	5-20 ng
Salt solution	1 µl
TOPO vector	1 µl
ddH ₂ O	to a final volume of 5 µl

The reaction was mixed gently and incubated at RT for 30 min, the time for incubation can be optimized to 1 h or overnight at 16°C. Then the reaction was used for *E. coli* transformation.

Gateway cloning (LR Reaction)

The following components were added to a 1.5 ml Eppendorf tube at RT and mixed gently, incubated at 25°C for 1 h, the time can be optimized to overnight at 16°C.

Table 2.9. Gateway LR reaction

Reagent	Volume
Entry clone (50-150 ng)	1-7 μ l
GATEWAY Binary Vector (150 ng)	1 μ l
LR Clonase II enzyme mix	2 μ l
TE buffer	to 10 μ l

After incubation, the reaction was terminated by adding 1 μ l of Proteinase K and incubated at 37°C for 10 min. Afterwards, the reaction was used for *E. coli* transformation.

Gibson Assembly

The Gibson Assembly method was performed according to protocol using NEB Gibson Assembly Cloning Kit (Gibson et al., 2009). DNA fragments were produced by PCR with primers designed to amplify the interested sequence containing 30-40 bp of overlapping sequences to the ends. Vectors were linearized with selected restriction enzymes. After confirming and purifying by agarose gel electrophoresis, DNA assembly reactions were prepared using 2-3-fold molar of DNA fragment to vector and 10 μ l Gibson Assembly Master Mix (2X) in 20 μ l total volume. Reactions were incubated in a thermocycler at 50°C for 50 min and transformed into *E. coli* competent cells.

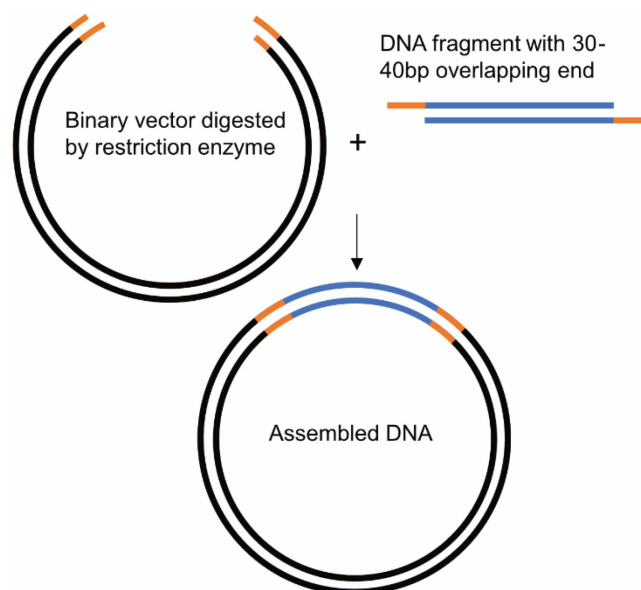


Figure 2.1. Visualization of Gibson Assemble workflow.

2.3.1.4 DNA sequencing

The plasmid DNA was sequenced by the Eurofin GATC Services referring to the requirement of the company. Sequencing results were evaluated by SnapGene software.

2.3.1.5 Transformation of *E. coli* and *Agrobacterium tumefaciens*

Escherichia coli

The *E. coli* DH5 α chemical competent cells mixed with plasmid DNA were incubated on ice for 10 min and heated at 42°C in a water bath for 90 s, then 700 μ l LB liquid medium was

added and incubated in the 37°C shaker with 200 rpm for 45 min. The 50–100 µl cells were spread on the LB plates with corresponding antibiotics and incubated overnight at 37°C.

Agrobacterium tumefaciens

The *A. tumefaciens* GV3101 electro competent cells were mixed with 1-10 ng plasmid DNA, then transferred into a pre-cooled sterile cuvette, and pulsed once with 1500V for 5 s by Electroporator 2510 (Eppendorf). Then 700 µl LB liquid medium was added, mixed gently, and incubated for 1.5 h in the 28 °C shaker with 200 rpm. The 50–100 µl cells were spread on the LB plates with corresponding antibiotics and incubated for 2 days at 28°C.

2.3.1.6 Plant genomic DNA extraction

The method for isolating plant genomic DNA was followed by the Edwards method. Plant tissue was frozen in liquid nitrogen (N₂) and grounded with a TissueLyser (Qiagen) in a 1.5 ml Eppendorf tube. After adding 400 µl extraction buffer, samples were placed at RT for 1 h. The samples were spun down at 13000 rpm for 10 min, 300µl supernatant was mixed with an equal volume of isopropanol and centrifuged at 13000 rpm for 10 min. After washing twice with 70% ethanol and drying in the fume hood, the pellet was dissolved in 100 µl H₂O.

Table 2.10. Edwards buffer preparation

Component	Volume
1 M Tris-HCl pH=7.5	20 ml
5 M NaCl	5 ml
0.5 M EDTA	5 ml
20% SDS	0.025 ml
ddH ₂ O	up to 100 ml

RNA isolation and cDNA synthesis

100 mg Arabidopsis leaf material was frozen in liquid nitrogen and ground with a pestle. Total RNA was extracted using NucleoSpin RNA Plus kit. RNA concentration and quality were determined by Nanodrop photometer with a 260/280 ratio and 260/230 ~2.0.

cDNA was synthesized with 1 µl RevertAid Reverse Transcriptase (Thermo Scientific), 4 µl 5×Reaction Buffer, 0.5 µl RiboLock RNase Inhibitor, 2 µl 2.5 mM dNTP, 0.1 -5 µg RNA template, 2 µl 10 µM Oligo(dt)₁₈. DEPC-treated water was added to adjust the total volume to 20 µl. The reaction was incubated at 42°C for 60 min and terminated by heating at 70°C for 10 min.

2.3.2 Biochemical methods

2.3.2.1 Plant protein extraction

30 mg plant leaf material was frozen in liquid nitrogen and grounded with a TissueLyser. Afterwards, 80 µl 1×SDS loading buffer was added to solubilize protein. The samples were

mixed completely by a vortex mixer (Vortex-Genie 2, Scientific Industries, Inc.) and heated at 95°C for 10 min by heat block. After spinning down for 10 min at 15000 rpm, the crude extraction was prepared for SDS-PAGE.

Table 2.11. 5×SDS loading buffer composition

250 mM Tris-pH6.8
10% SDS
0.05% Bromphenol blue
50% glycerol
0.5 M DTT

2.3.2.2 SDS-PAGE

The SDS-PAGE preparation was followed by the protocol of Laemmli(Laemmli, 1970). 8% of separation gels were used for electrophoresis. 20 µl samples and 3 µl PageRuler Prestained Protein Ladder were loaded for running at 120 v 2 hours in the Mini PROTEIN 3 system (Biorad). When the red band of PageRuler (approx. 70 kDa) had run to the bottom of the gel, the gel should be stopped and prepared for blotting.

2.3.2.3 Western blot analysis

The nitrocellulose (NC) membrane (Cytiva), filter papers (Blotting papers, 0.75 mm Carl Roth), and gels were soaked in 1 × transfer buffer and combined as sandwich on the PerfectBlue semi-dry transfer blotter (PeqLab, Erlangen) with the sequence of 2 pieces of filter papers, gel, NC membrane, and 2 filter papers. Blotting was set as 200 mA/gel for 1 h.

After blotting, the NC membrane was rinsed in water and stained with Ponceau S Staining Solution 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid to visualize the successful transfer of proteins. The unspecific binding sites were blocked by 5% milk TBS-Tween 20 at RT on a shaker for 1 h.

Before incubating with antibodies, the membrane was washed with TBS-Tween 20 solution 2 times for 5 min and then added with 10 ml 5% milk TBS-Tween 20 with corresponding primary antibody to bind the interest proteins at 4°C overnight on a shaker. To remove the extra primary antibody, the membrane was washed with TBS-Tween 20 solution 3 times on a shaker for 10 min. After washing, the membrane was incubated with 10 ml 5% milk TBS-Tween 20 with a secondary antibody according to the species of primary antibody for 1 h at RT on the shaker. The membrane was washed 3 times with TBS-Tween 20 solution to flush away the redundant secondary antibody and kept with ECL Detection Reagents buffer for 5 min before exposure by CCD camera (Amersham Imager600 detection system) to show the proteins and marker bands.

Table 2.12. Antibodies used in Western Blot

Antibodies	Dilution	Immunogen	Type
α -GFP	1:5000	mouse	Primary antibodies
α -Myc	1:5000	mouse	
α -HA	1:3000	mouse	
HRP-Conjugated Antibodies	1:10000	mouse	Secondary antibody

2.3.3 Plant transformation

2.3.3.1 Transient expression in *N. benthamiana*

Agrobacterium-mediated transient transformation of *N. benthamiana* was performed for transient expression protein to examine receptors expression and immune response. GV3101 carrying binary constructs was streaked out on the LB medium with corresponding antibiotics for 2 days, a single colony was picked and incubated in LB liquid medium with selective antibiotics in the 28°C shaker with 200 rpm overnight. After spinning down the culture, the pellet was washed with 10 mM MgCl₂ and 150 μ M acetosyringone. The suspension was adjusted to OD₆₀₀=0.2 to 0.3 and set aside for 2 h at RT in the dark. Then the mixture was infiltrated by a syringe into the leaves of 4 weeks old *N. benthamiana*. After 2-day infiltration, the plant tissue was used for subsequent experiments.

2.3.3.2 *Agrobacterium*-mediated stable transformation

Arabidopsis thaliana

The GV3101 was grown in a 3 ml LB liquid medium with appropriate antibiotics in the 28°C shaker at 200 rpm overnight. 2 ml culture was inoculated into a 200 ml LB liquid medium with appropriate antibiotics and incubated continually overnight. Spinning down the pellet at 5000 rpm for 15 min in RT, the cells were resuspended with 200 ml ½ MS liquid medium. 6-8 weeks old *Arabidopsis* plants were dipped into the infiltration medium for 90 s and covered with plastic bags to maintain humidity for 36 h, then put back to the greenhouse.

T0 seeds were collected and screened with corresponding antibiotics. To screen seeds on ½ MS medium with antibiotics, the seeds were surface sterilized firstly by chlorine gas (3 ml concentrated HCl + 40 ml Sodium-hypochlorite solution) in the desiccator for 6 h, then placed in the sterile bench to release chlorine gas for 1 h and spread on ½ MS medium finally. The plates were incubated in the Phyto chamber with lids upwards.

Table 2.13. ½ MS liquid medium (1 L) preparation

MS	2.1 g
Sucrose	50 g
Silvet L-77	500 μ l
150 mM Acetosyringon	400 μ l

Nicotiana benthamiana

Material and Methods

The transformation steps should be performed in a sterilized environment.

After sterilizing *N. benthamiana* seeds, the plants were grown in the glassware for 7-8 weeks in the Phyto chamber (23°C, 60% humidity, 13 h light). *Agrobacterium*-carrying constructs were incubated in the LB medium with corresponding antibiotics. *Agrobacteria* suspension was adjusted to OD₆₀₀=1 and resuspended with 10 mM MgCl₂. Plant leaves were cut into 1 cm² pieces and incubated with *agrobacteria* suspension for 3 min, then put on the plates with MS2% medium and incubated for 2 days at RT in darkness.

Leaf pieces were washed in the 10 mM MgSO₄ containing cefotaxime, then transferred into Selection Medium every 10 days until callus formation.

After 7-8 weeks, the shoots were cut and transferred to Root Medium. Once the roots formed, the plants were transferred into the soil and grown in the greenhouse.

Solanum lycopersicum

The transformation steps should be performed in a sterilized environment.

S. lycopersicum m82 seeds were sterilized and sow into glassware for 10 days before transformation in the dark at 22°C, after got enough cotyledons, the cotyledons were cut to remove both ends and stab a little hole in the middle of the vein with drops of Liquid Germination medium. The leaves were placed on the plates with the Conditioning medium for 2 days in the dark at 22°C.

Agrobacterium was prepared and adjusted to OD₅₉₀=1 with 10 mM MgSO₄ and 150 μM acetosyringon. The cotyledons with 2 drops of *agrobacterium* suspension were incubated for 2 days in the dark at 22°C.

The cotyledons were transferred to the Selection medium and kept in the Phyto chamber (23°C, 14 light, 50% humidity). After 7-8 weeks, the shoots were cut and transferred to the Rooting medium. Once the roots formed, the plants were transferred into the soil and grown in the greenhouse.

Solanum tuberosum

The transformation steps should be performed in a sterilized environment.

S. tuberosum Désirée plants in the glassware were cultivated without contamination by Caterina Brancato, 3-4 weeks old plant leaves were cut and stab two little holes in the middle of vein, co-cultivated with *Agrobacterium* which pre-prepared in YEB medium in the petri dishes for 3-5 min on a shaker at 80 rpm. The leaves with *agrobacteria* suspension were incubated in dark at RT for 2-3 days.

Leaves were placed into callus induction medium with leaf surface facing down. After 1 week, leaves were moved to selection medium for shoots formation. After a few weeks, the shoots were cut and transferred to Rooting medium. When the roots formed, the plants were transferred into soil to the greenhouse.

2.3.3.3 Crossing

Arabidopsis is a self-pollination plant, so at the stage of developed inflorescences, the mature siliques, open flowers, and buds with white tips on mother plants should be removed, only 1 appropriate right-size bud was kept in the inflorescence, with a fine-tipped forceps, the sepal, petal, and immature stamen were removed carefully, only stigma was kept. The stamen on father plant was taken out from the open flower and nipped carefully to touch the stigma of mother plant for pollination. This pollination step can be repeated 2 days later to make sure the emasculated inflorescences get anthers. The pistils were marked with stickers around the stems. After 15-25 days, the siliques were ready to be harvested when they turned to light brown before opening.

2.3.4 Bioassay

2.3.4.1 ROS assay

The leaves were cut into pieces of 0.3 cm × 0.3 cm size and equilibrated in the petri dishes with ddH₂O overnight. One leaf piece was gently transferred by pipet tip into one well of a 96-well plate with 80 µl ddH₂O and 10 µl mastermix (1 ml: 4 µl 5 mg/ml peroxidase, 10 µl 20 mM luminol L-012, 986 µl ddH₂O), and incubated for 40 min in darkness. The luminol-chemiluminescence reaction was measured after adding 10 µl elicitor pre well by 96 well Luminometer (Mithras LB 940, Berthold Technologies). The data was exported from the software Microwin and preliminarily analyzed by Excel.

2.3.4.2 Ethylene assay

Leaves were cut into pieces of 0.4 cm × 0.4 cm size and incubated in the petri dishes with ddH₂O overnight. Three leaf pieces were placed into 6 ml glass tubes with 400 µl MES buffer (10 mM, pH to 5.7), after being added with respective elicitors, the glass tubes were sealed with rubber plugs and incubated on a shaker for 4 h at 170 rpm. 1 ml air sucked from one glass tube was injected into the flame ionization detector of a gas chromatograph (GC-2014, Shimadzu, Duisburg) by 1 ml syringe with a needle. The values of ethylene were calculated based on the area according to the retention time of ethylene.

2.3.4.3 Hypersensitive response (HR)

4–5-week-old *N. benthamiana* or 5-week-old *Arabidopsis* leaves were infiltrated with 20 µM xylanase. The *N. benthamiana* leaves and *Arabidopsis* leaves were photographed for cell

death under UV light using an Amersham ImageQuant 800 and an integrated Cy5 filter (GE Healthcare; Chalfont St. Giles, UK) at 7 or 3 dpi, respectively.

2.3.4.4 Pathogenicity assays

Pseudomonas syringae infection

5-6-week-old *Arabidopsis* and 4-week-old *N. benthamiana* plants were used for infection assay. *P. syringae* infection was performed according to the protocol (Yao et al., 2013). *P. syringae* pv. *tomato* (*Pst*) DC3000 or *P. syringae* pv. *tabaci* (*Pstab*) was streaked out from -80 °C freezer stock on the King's B medium containing 50 µg/ml Rif and incubated at 28°C for 2 days. A single colony was inoculated in 3 ml liquid King's B culture with 50 µg/ml Rif and incubated in a 28°C shaker for 12 h. The bacteria were harvested by centrifugation at 4000 rpm for 5 min at RT and washed once with 10 mM MgCl₂ buffer. After resuspending in 10 mM MgCl₂ buffer, the optical density (OD) of the cell suspension was measured with a spectrophotometer which was set to 600 nm. The concentration of bacteria for infection was followed by Table 2.11 and depended on the method of inoculation. For *Pst* DC3000, an OD₆₀₀=0.2 should be approximately 1×10⁸ CFU/ml.

Table 2.14. Concentrations of bacteria and sampling time applied in *Pseudomonas* infection

Inoculation methods and plants	Inoculation concentration (CFU/ml)	Sampling time
Dip or spray <i>A. thaliana</i>	1 ×10 ⁶	3 dpi
Syringe infiltration <i>A. thaliana</i>	1 ×10 ⁴	3 dpi
Syringe infiltration <i>N. benthamiana</i>	1 ×10 ⁴	4 dpi

For bacteria syringe inoculation, the bacterial suspension was gently infiltrated into leaves on the abaxial surface with a 1 ml needleless syringe. The 4th and 5th *N. benthamiana* leaves and 9th, 11th, and 13th *A. thaliana* leaves were used for infiltration.

For spray inoculation, the concentration of bacterial suspension was adjusted to 10⁶ CFU/ml with 0.02% Silwet L-77 in an ethanol-sterilized spray bottle. Until the surface of the rosettes was totally covered with little beads of bacteria, the leaves were saturated.

In both infiltration and spray inoculation, the leaves were left to dry and then covered with transparent plastic lids to maintain high humidity in the Phyto chamber.

After 2-4 days, the leaf discs were harvested with a 0.5 cm² disposable biopsy punch, surface sterilized in 70% ethanol for 15 s, and rinsed in water. Subsequently, the leaf discs were dried and placed into a 1.5 ml Eppendorf tube with 3 mm borosilicate glass beads and 200 µl 10 mM MgCl₂. All the samples were ground with a TissueLyser (Qiagen) until the tissue was totally smashed. The samples were diluted in 10 mM MgCl₂ in a series (1:10, 1:100, 1:1000, 1:10000, xxx). 10 µl of each dilution series was spotted on the King's B medium

containing appropriate antibiotics. The plates were incubated at 28 °C for approximately 30 h until the colonies were visible to be counted.

During the infiltration of *Pstab* in *N. benthamiana*, the *Pstab* suspension was infiltrated into the marked circle area. After 4 d, cell death caused by *Pstab* was photographed under UV light using an Amersham ImageQuant 800 and an integrated Cy5 filter (GE Healthcare; Chalfont St. Giles, UK).

Botrytis cinerea infection

Spores of *B. cinerea* strain B05.10 were diluted in potato dextrose broth to the final concentration of 2×10^6 spores/ml and 4 μ l of spore suspension was dropped on the 5-6-week-old *A. thaliana*; 2 μ l 10^4 spores/ml was dropped on 4–5-week-old tobacco. 3 leaves were inoculated on each plant. After the infection, all plants were kept under the sealed transparent hood at high humidity. 2 days after inoculation, lesion diameters were measured with Image J software or Photoshop CS5 Lasso tool.

Phytophthora infection

Phytophthora capsici and *Phytophthora infestans* were inoculated on leaves of *N. benthamiana* in the form of hyphae plugs which were collected from medium places by 0.5 cm² disposable biopsy punch. The infected leaves were incubated in 245 mm square dishes with high humidity for 2 days without light. Pictures were taken by Canon EOS 80D under UV light using 100W longwave UV lamp (Analytik Jena US). Lesion diameters on leaves were analyzed with Image J software.

Albugo laibachii infection

10^5 spores/ml zoospore suspension was sprayed on 3-week-old plants covered in a plastic bag with an airbrush gun (Conrad Electronics GmbH), in average every plant needed to be sprayed with 1 ml suspension. Then plants in the bag were placed in the 4°C cold room without light overnight to boost spores' germination. Then the plants were uncovered and incubated under 21°C for 10 h and 16°C for 14 h conditions. At 9 dpi, symptoms occurred and were recorded on both adaxial and abaxial sides by Canon EOS 80D.

2.3.4.5 Biomass of *N. benthamiana*

Aerial parts of 4-week-old plants were sampled and weighed for wet weight measurement. Then aerial parts were covered by tin foil and dried in the oven at 100°C for 20 min, then 70°C for 12 h until weight was constant to measure dry weight.

To measure seed weight, three mature tobacco seed pods of every plant were taken and weighed together as one transgenic line or wild-type seed weight.

Material and Methods

The germination of *N. benthamiana* seeds was recorded after seeds were sowed into the soil and grew in the greenhouse for 7 days. Seedlings were taken by camera Canon EOS 80D.

2.3.5 Software and Statistics

DNA sequence alignment and primer design were executed by SnapGene. All graphs were plotted using GraphPad Prism 8. Lesion diameters and PCR gel images were conducted by Image J. Images were combined and optimized with Affinity Designer. Data analysis was carried out by IBM SPSS Statistics 27 and GraphPad Prism 8.

3 Results

3.1 Stacking PRR gene expression in Arabidopsis

Since the interfamily transfer of EFR into crops contributes to enhanced resistance to bacterial infection as well as gene stacking strategy is a promising biotechnology to achieve broad range resistance (Rodriguez-Moreno et al., 2017), stacking multiple PRRs in crops to gain broad-spectrum resistance to pathogens is imperative in plant breeding. It is a long-term procedure to obtain stable transformants in crops, therefore, model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* were used firstly to test this hypothesis. To achieve broad-spectrum resistance to distinct pathogen genera, the tomato (*Solanum lycopersicum*) RLK SICORE recognizing csp22 derived from bacterial cold-shock protein, the RLP SIEIX2 recognizing xylanase secreted by fungal pathogens and the potato (*Solanum microdontum*) RLP SmELR perceiving oomycete INF1 were chosen for building these PRR gene cassette in one binary vector which was used for transformation in *A. thaliana*.

3.1.1 Construction of a three receptor gene-cassette *SICORE-SmELR-SIEIX2*

The coding sequences of SICORE, SmELR, and SIEIX2 retrieved from NCBI were firstly cloned into the corresponding binary vectors (pB7FWG2.0::*SICORE*, pGWB20::*SmELR*, and pGWB14::*SIEIX2*, respectively), which are driven by 35S promoter and C-terminally fused with different tags (Figure 3.1a). Subsequently, 35S-*SmELR-Myc* cassette was PCR amplified and assembled into the *Apal* site of pB7FWG2.0::*SICORE* by Gibson assembly, yielding two gene-cassette plasmid pB7FWG2.0::*SICORE-eGFP::SmELR-Myc* (Figure 3.1b). To avoid confusing of plant to use one promoter (e.g. 35S promoter) for the expression of multiple genes, different promoters were used for the three gene-cassette assembly, in which *SICORE* and *SmELR* were driven by 35S promoter whereas expression of *SIEIX2* was under the control of the *UBQ10* promoter. To achieve such assembly (pB7FWG2.0::*SICORE-eGFP-SmELR-Myc-UBQ10-SIEIX2-HA*), *UBQ10* promoter and *SIEIX2-HA* cassette were PCR amplified using pRW004-SM and pGWB14::*SIEIX2* as templates, respectively, and assembled into the *PmeI* site of pB7FWB2.0::*SICORE-eGFP::SmELR-Myc* (Figure 3.1c).

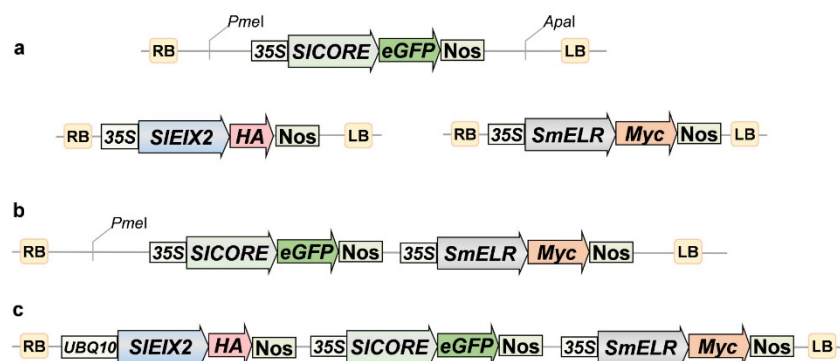


Figure 3.1. Schematic representation of gene cassette construction.

a, In the single receptor gene expression vectors, *SICORE* was cloned into pB7FWG2.0 under the control of 35S promoter and with C-terminal *eGFP*-fusion; *SmELR* was cloned into pGWB20 under 35S promoter and with C-terminal *Myc*-fusion; *SIEIX2* was cloned into pGWB14 under 35S promoter and with C-terminal *HA*-fusion by gateway cloning method. **b**, After digesting plasmid pB7FWG2.0::*SICORE* by *Apal* restriction enzyme, the fragment 35S-*SmELR*-*Myc*-*Tnos* amplified using specific primers (Table 2.4) was assembled to linearized pB7FWG2.0::*SICORE* by Gibson Assembly method. **c**, The three genes cassette was constructed by linearized pB7FWG2.0::*SICORE*-*SmELR* using *PmeI* enzyme and two fragments *UBQ10* and *SIEIX2*-*HA*-*Tnos* amplified with specific primers utilizing Gibson Assembly to stack these three receptor genes into one vector.

3.1.2 Transient expression of gene cassettes in *Nicotiana benthamiana*

To determine whether the constructs obtained above are functional, the binary vectors pB7FWG2.0::*SICORE*, pGWB20::*SmELR*, pB7FWG2.0::*SIEIX2*, pB7FWB2.0::*SICORE*-*SmELR*, and pB7FWB2.0::*SICORE*-*SmELR*-*SIEIX2* (hereafter CORE, ELR, EIX2, CORE-ELR, CORE-ELR-EIX2) were transformed into *A. tumefaciens* strain GV3101 competent cells by electroporation. The transformants harboring the corresponding plasmid were used for transient expression assay in *N. benthamiana*. After agroinfiltration into *N. benthamiana* leaves, the leaves were treated with 100 nM csp22 for ROS burst measurement or with 200 nM xylanase for ethylene accumulation assay. The results showed that expressing either CORE alone or gene cassettes (CORE-ELR and CORE-ELR-EIX2) containing CORE confer clear ROS burst upon csp22 treatment (Figure 3.2a). *N. benthamiana* transiently expressing EIX2 alone or the gene cassette CORE-ELR-EIX2 induced ethylene production upon xylanase treatment, whereas untransformed *N. benthamiana* leaves produced no ethylene (Figure 3.2b). SCP^{Ss} which is recognized by NbRE02 receptor from *N. benthamiana* served as a positive control in ethylene measurement (Yang et al., 2023). To detect the expression levels of these receptors, total proteins were extracted from 30 mg infiltrated leaves and loaded in the SDS-PAGE gels. The protein levels were detected by Western blot with the corresponding antibodies (Figure 3.3b). These results demonstrate that the two gene- and three gene-cassettes are functional as the single-gene construct and can be used for stable transformation into *A. thaliana*.

Results

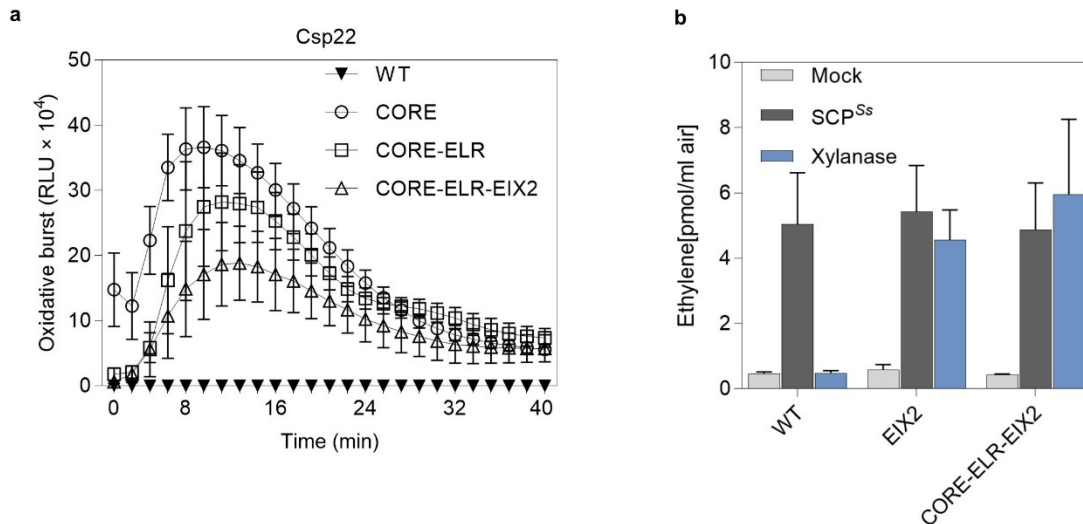


Figure 3.2. Immune responses in *N. benthamiana* leaves transiently expressing CORE-ELR-EIX2 cassette.

a, ROS burst in *N. benthamiana* leaf discs transiently transformed CORE, CORE-ELR, or CORE-ELR-EIX2 receptors and treated with water (mock) or 100 nM csp22. Curves show luminol-dependent light emission in 40 min. Values and error bars show means \pm S.D. of three replicates. **b**, Ethylene production in *N. benthamiana* leaf discs transiently expressed EIX2, or CORE-ELR-EIX2 receptors 4 h after treatment with water (mock), 500 nM SCP^{Ss} (positive control) or 200 nM xylanase. Bars represent means + S.D. of three replicates.

3.1.3 Stable transformation of single genes and multiple-gene cassettes in *A. thaliana*

To obtain stably transformed receptors in *A. thaliana*, the plasmids containing either single receptor or 2- or 3-gene cassettes were transformed into *A. thaliana* Col-0 background by *Agrobacterium*-mediated transformation. T1 generation plants were obtained by selecting the T0 seeds with the corresponding antibiotics. Subsequently, the presence of transformed genes was confirmed by genomic DNA PCR analysis and the protein levels of each receptor were determined by Western blot assay (Figure 3.3a and b). The positive T1 transgenic lines were maintained to harvest the seeds, and the yielding T2 generation transformants were screened on the selection medium. The antibiotic-resistant seedlings were used for immune response analysis and pathogen inoculation analysis. The detectable protein accumulation of each receptor demonstrates that the receptors are expressed well in Arabidopsis and the functions of receptors are waiting for corroboration. All transformants showed normal growth through an entire growth cycle, therefore two independent transgenic lines from each transformation were used for further studies.

Results

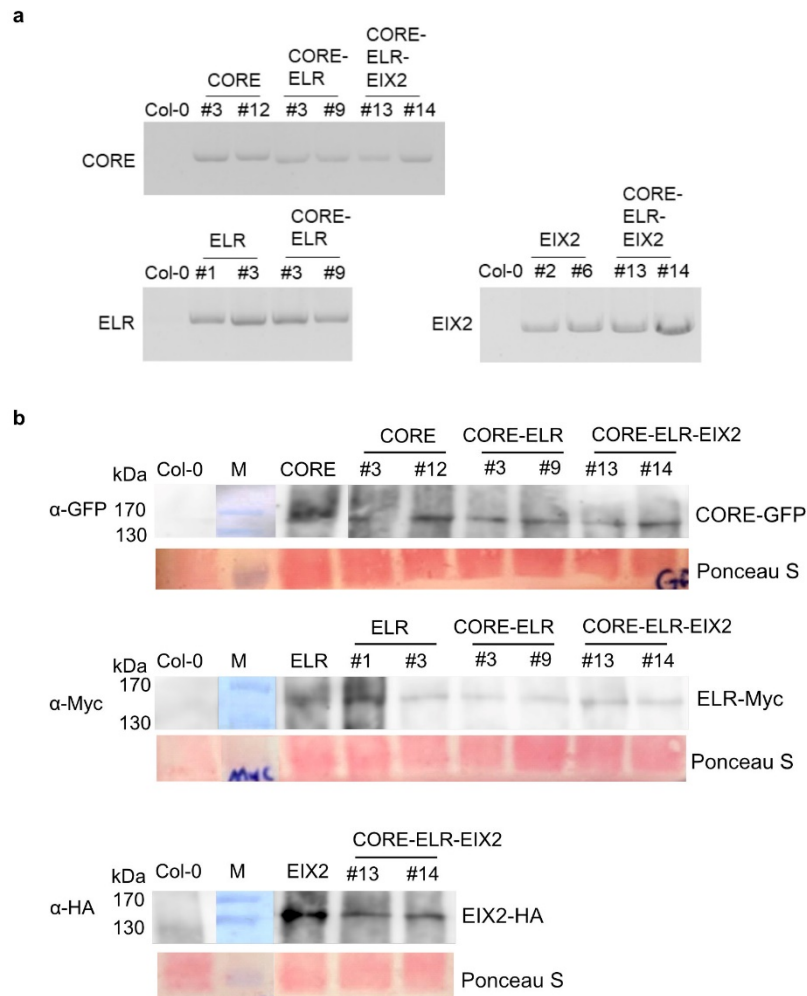


Figure 3.3. Characterization of receptor stable transgenic lines in *A. thaliana*.

a, PCR amplification of *SICORE*, *SmELR*, and *SIEIX2* gene fragments using genomic DNA extracted from T1 generation leaves transformed with CORE, ELR, EIX2, CORE-ELR, CORE-ELR-EIX2. The genomic DNA of Col-0 served as a negative control. **b**, The protein accumulation of CORE-GFP, ELR-Myc, and EIX2-HA in T1 generation leaves was determined by Western blot with the corresponding antibodies. Total protein from Col-0 (left side of marker, M) and proteins from transient expression of receptors (right side of the marker) served as negative and positive controls, respectively.

3.1.4 Immune responses in *A. thaliana* transgenic lines

Upon ligand perception, pattern recognition receptors trigger a set of immune responses, such as Ca^{2+} influx, ROS burst, MAPK activation, ethylene production, and defense-related gene expression. These responses act synergistically and collectively providing resistance to pathogens. In this section, ROS burst, ethylene accumulation, and hypersensitive response assays are performed using the T2 transgenic lines treated with the corresponding ligands.

3.1.4.1 ROS burst in CORE-expressing transgenic Arabidopsis

To investigate whether the CORE-expressing *A. thaliana* lines are responsive to csp22, the selected T2 transgenic lines with single receptors and gene cassettes as well as Col-0 plants were treated with water (mock), 100 nM flg22, or csp22, and the ROS burst was measured in a time course of 45 min. All the transgenic plants and Col-0 showed ROS accumulation albeit

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to different levels after flg22 treatment. However, only the transgenic lines expressing CORE showed ROS burst upon csp22 treatment, whereas Col-0 and transgenic lines not expressing CORE were insensitive to csp22 (Figure 3.4). These results demonstrate that CORE confers ROS response to csp22, and multiple gene stacking constructs have no effect on CORE function.

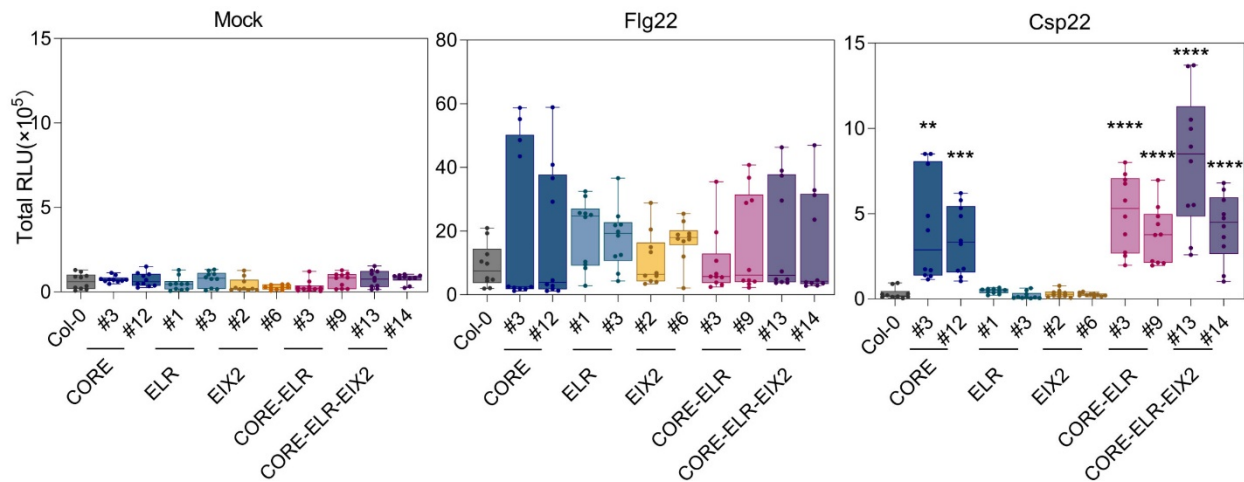


Figure 3.4. The ROS production in transgenic Arabidopsis.

Leaf discs from Col-0 and transgenic lines were treated with MilliQ water (mock, as a negative control), 100 nM flg22 (as positive control), or csp22, and the total oxidative burst was summed in 45 min. Data points are indicated as dots from three independent experiments (n=10). Box plots show the minimum, first quartile, median, third quartile, and maximum of total ROS burst. Asterisks represent significant differences to Col-0 treated with csp22 by two-tailed Student's t-test (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

3.1.4.2 ROS burst upon xylanase treatment in Arabidopsis

To determine whether EIX2-expressing Arabidopsis plants can cause ROS burst upon xylanase treatment, the T2 transgenic lines EIX2 # 2 and # 6, and Col-0 plants were treated with water (mock), 5 μ M xylanase, and the ROS burst was measured in a time course of 240 min. The results show that xylanase can trigger ROS production not only in EIX2 transgenic lines but also in Col-0 plants at the early time (40 min), furthermore cause a long-lasting second phase of ROS burst for 3 h, indicating that there must be an essential component existing in the immune system of Arabidopsis to respond to xylanase (Figure 3.5a). To identify the component associated with xylanase perception in Arabidopsis, firstly the mutants of ETI signaling pathway, *eds1*, *pad4*, *adr1 triple*, *nrg1 double*, *helperless*, and Col-0 plants were treated with 5 μ M xylanase. Consistent with the ROS burst in Col-0, the mutants^{ETI} showed similar two phases of ROS production (Figure 3.5b). Next, the 54 *rlp* mutants, *sobir1* mutant, and *efr fls2 cerk1 lym3* quadruple mutants were tested for ROS production upon xylanase treatment (Supplementary Figure 7.1). The results showed that *rlp6* and *rlp13* mutants have no ROS response to xylanase (Figure 3.5c), while *sobir1* mutant

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and *efr fls2 cerk1 lym3* quadruple mutant still respond to xylanase. These results indicate that the ROS burst caused by xylanase in Arabidopsis is associated with RLP6 and RLP13.

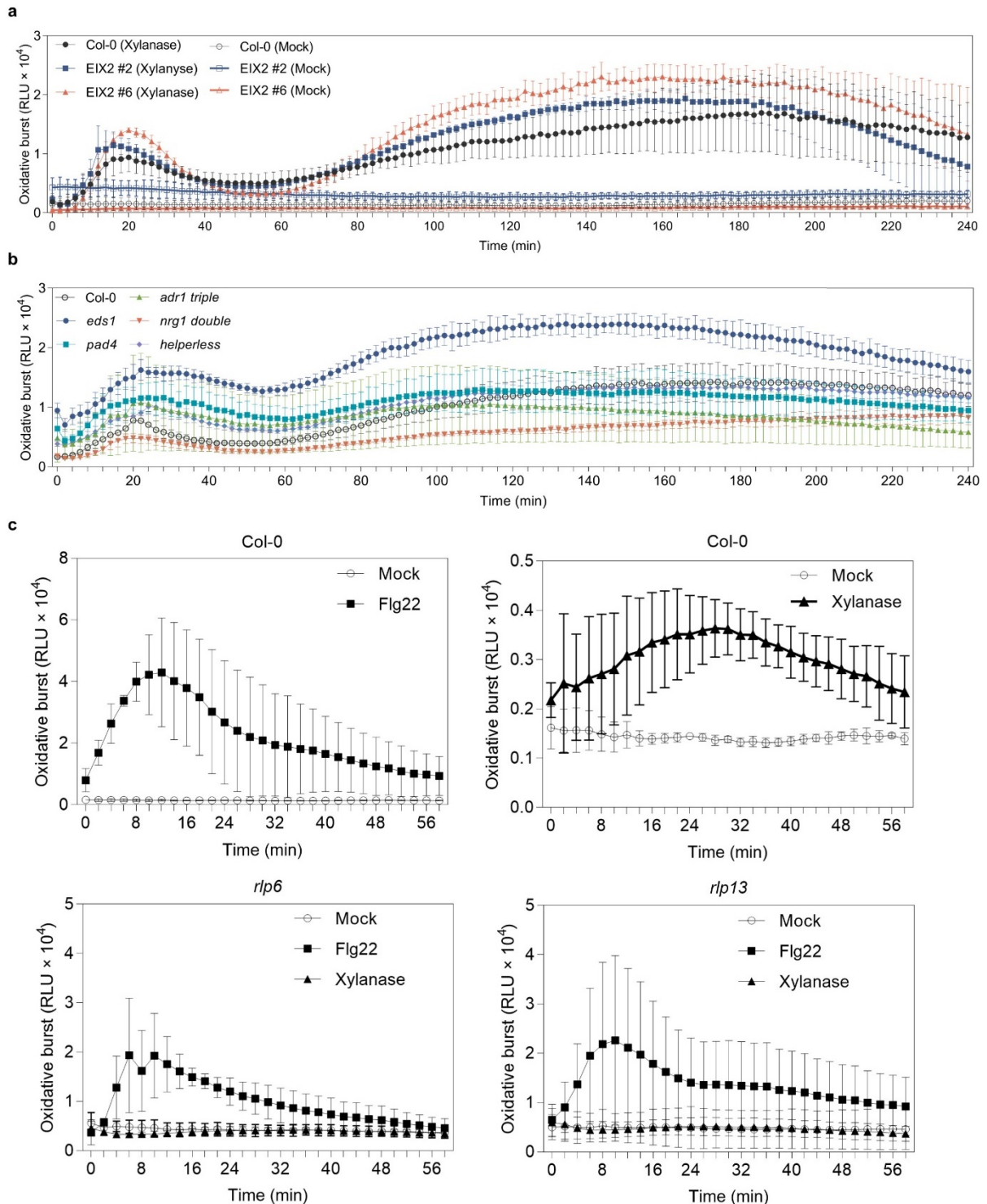


Figure 3.5. ROS production triggered by xylanase in Arabidopsis.

a,b, Leaf discs from Col-0, (a)EIX2 transgenic lines, and (b)mutants were treated with MilliQ water (mock, as a negative control), 5 μ M xylanase, and the curves show luminol-dependent light emission in 240 min. Values and error bars show means \pm S.D. of three replicates. **c**, ROS burst in Col-0, *rlp6*, and *rlp13* leaf discs treated with water (mock), 100 nM flg22 (as a positive control), or 5 μ M xylanase. Curves show luminol-dependent light emission in 60 min. Values and error bars show means \pm S.D. of three replicates.

3.1.4.3 Ethylene production in CORE/EIX2-expressing transgenic Arabidopsis

Because *csp22* and xylanase can induce ethylene biosynthesis upon CORE and EIX2 recognition, respectively in tomato (Jehle et al., 2013; L. Wang et al., 2016), whether *A. thaliana* transgenic lines expressing CORE and EIX2 produce ethylene upon elicitation was investigated. The plant leaf discs were treated with water (mock), 1 μ M flg22 (as positive control), 2 μ M *csp22*, or 200 nM xylanase and incubated for 4 h before ethylene measurement. All the plants were responsive to flg22, whereas only the CORE-expressing and EIX2-expressing plants were responsive to *csp22* and xylanase, respectively (Figure 3.6). While other transformants like ELR expressing lines caused no ethylene production to neither *csp22* nor xylanase. These results suggest that ectopic expression of a single receptor CORE or EIX2, or stacking receptors CORE-ELR or CORE-ELR-EIX2 is functional in Arabidopsis.

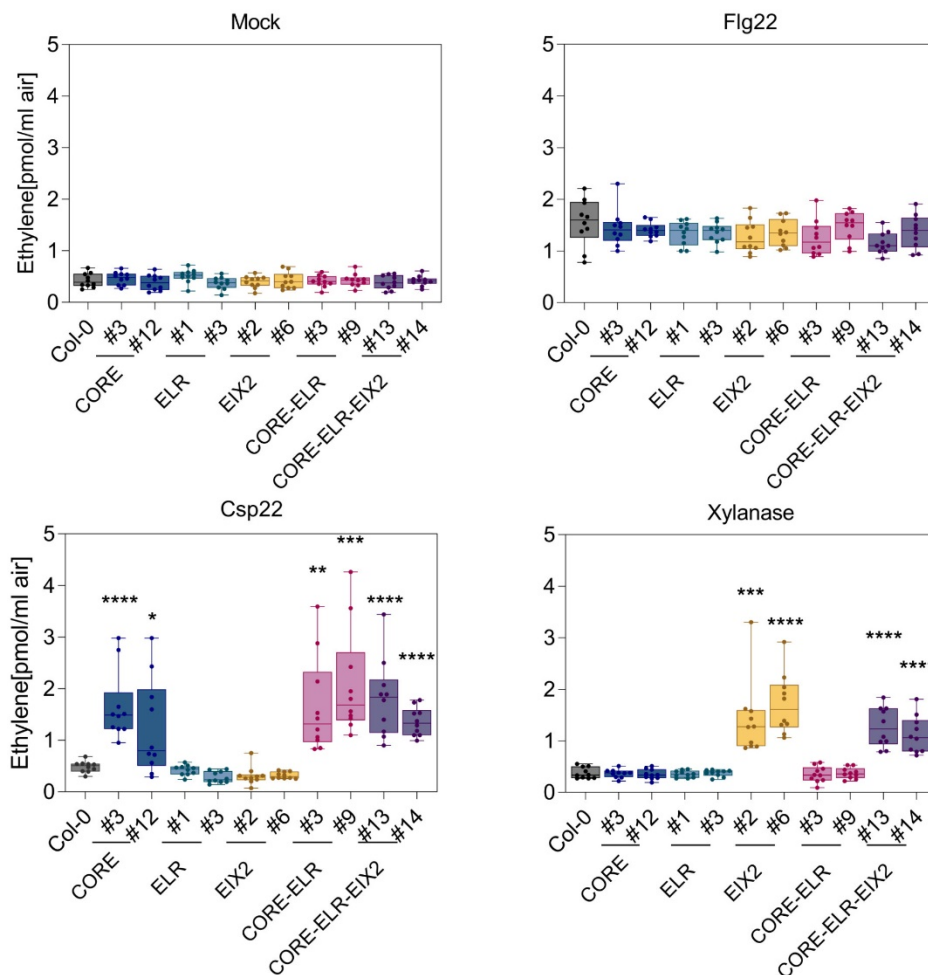


Figure 3.6. Ethylene accumulation in transgenic Arabidopsis.

Leaf discs from Col-0 and transgenic lines were treated with MilliQ water (mock, as a negative control), 1 μ M flg22 (as a positive control), 2 μ M *csp22*, or 200 nM xylanase and incubated on a shaker for 4 h before measurement. Data points are indicated as dots from three independent experiments (n=10). Box plots show the minimum, first quartile, median, third quartile, and maximum of ethylene production. Asterisks indicate significant differences to Col-0 treated with *csp22* or xylanase by two-tailed Student's t-test (* $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

3.1.4.4 Hypersensitive response in EIX2-expressing transgenic Arabidopsis

Xylanase induces cell death in EIX2-expressing transgenic *N. tabacum* cv Samsun leaves, whereas patterns rarely trigger cell death except for pg13 recognized by RLP42 in Arabidopsis (Ron & Avni, 2004; Zhang et al., 2021; Zhang et al., 2014). To investigate whether xylanase can cause cell death in EIX2-expressing Arabidopsis plants, 20 μ M xylanase was infiltrated into those plant leaves. After 3 d infiltration, EIX2-expressing plants, but not Col-0, showed clear cell death upon the infiltration area (Figure 3.7). It demonstrates that xylanase triggers cell death in Arabidopsis through EIX2 recognition. It suggests that the signal components required for EIX2-triggered cell death are conserved in Arabidopsis and *N. tabacum*.



Figure 3.7. Cell death in leaves of EIX2-expressing Arabidopsis.

Leaves were infiltrated with 20 μ M xylanase and visualized at 3 d post infiltration.

3.1.5 Expression of single receptors and stacking receptors in Arabidopsis confers broad-spectrum resistance to pathogens

Interfamily transfer of EFR enhancing resistance to bacteria provides an example for PRRs transformation among plant species (Lacombe et al., 2010), which might be suitable to gain broad-spectrum resistance to variable pathogens. To test this hypothesis, Solanaceae PRRs CORE, ELR, and EIX2, recognizing PAMPs derived from bacteria, oomycete, and fungi, respectively, are chosen for stacking and transforming into Arabidopsis. Since the individual receptors and stacking receptors are functional in Arabidopsis, the resistance of these transformant lines was investigated against different pathogens.

3.1.5.1 CORE-expressing lines enhance resistance to *P. syringae* pv. *tomato* (*Pst*) DC3000

To test the resistance to bacteria, those transgenic Arabidopsis lines were inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000. Three days post infiltration of *Pst* DC3000 in leaves, bacterial growth was restricted in CORE-expressing transgenic plants compared to Col-0 and other transgenic lines without expressing CORE (Figure 3.8). Interestingly, both CORE-ELR-expressing and CORE-ELR-EIX2-expressing lines exhibited similar bacterial growth to the single CORE-expressing plants indicating that ELR and EIX2 as oomycete and fungal PAMP-recognizing receptors have no effect on CORE function.

Results

Similar results were obtained when using the spray inoculation methods (Supplementary Figure 7.2).

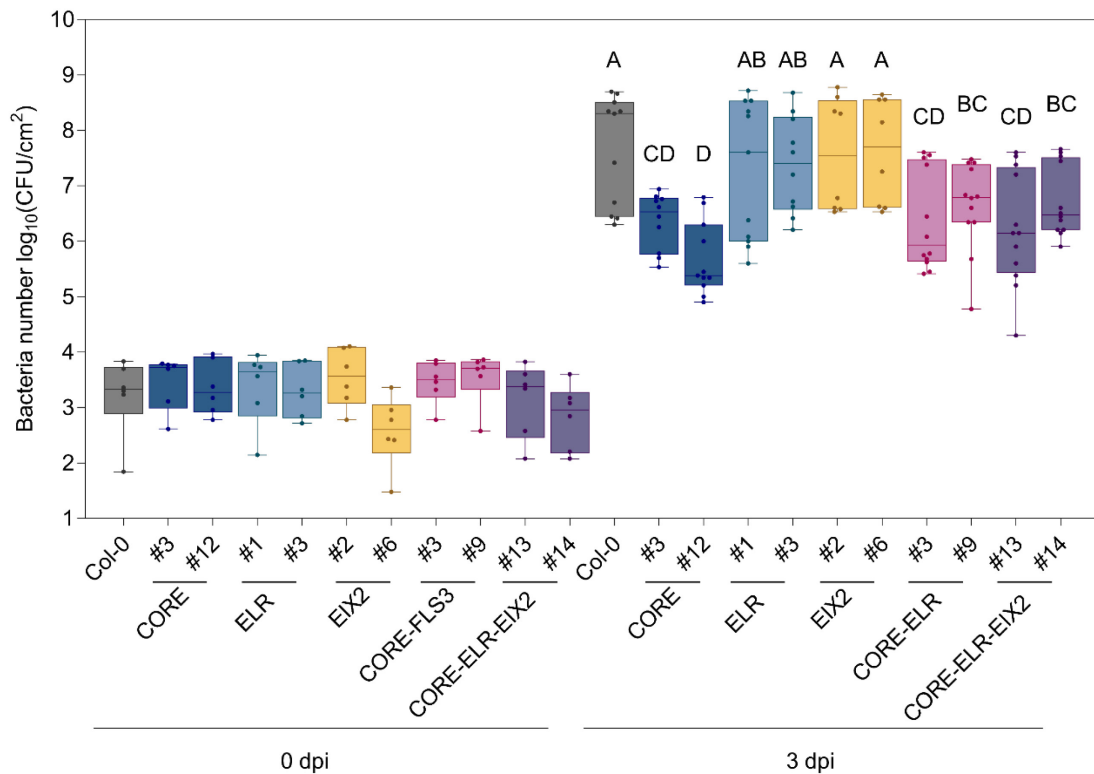


Figure 3.8. CORE-expressing Arabidopsis plants enhance resistance to bacterial pathogen *Pst* DC3000.

Col-0 and transgenic lines expressing CORE, ELR, EIX2, CORE-ELR, and CORE-ELR-EIX2 were infiltrated with 1×10^4 cfu/ml *Pst* DC3000. Bacteria were quantified in extracts of leaves at 0 and 3 d post-infiltration. Data points are indicated as dots from three independent experiments (0 dpi, n=6; 3 dpi, n=11). Box plots show the minimum, first quartile, median, third quartile, and maximum of log₁₀ cfu/cm² leaf tissues. Different letters above the box blot at 3 dpi indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$).

3.1.5.2 EIX2-expressing lines enhance resistance to *Botrytis cinerea*

To test the resistance to the fungal pathogen, those transgenic Arabidopsis lines were inoculated with the necrotrophic pathogen *Botrytis cinerea* B05.10. After 2 day-infection, the lesion sizes were significantly smaller on EIX2- and CORE-ELR-EIX2-expressing lines compared to those on Col-0 and the lines without expressing EIX2 (Figure 3.9). Notably, when the infected leaves were incubated over 3 days, the whole leaf area was fully colonized by grey mycelium and conidiation of *B. cinerea* was observed on all the lines tested. These results suggest that EIX2 provides certain resistance to *B. cinerea* merely at the early stage of infection.

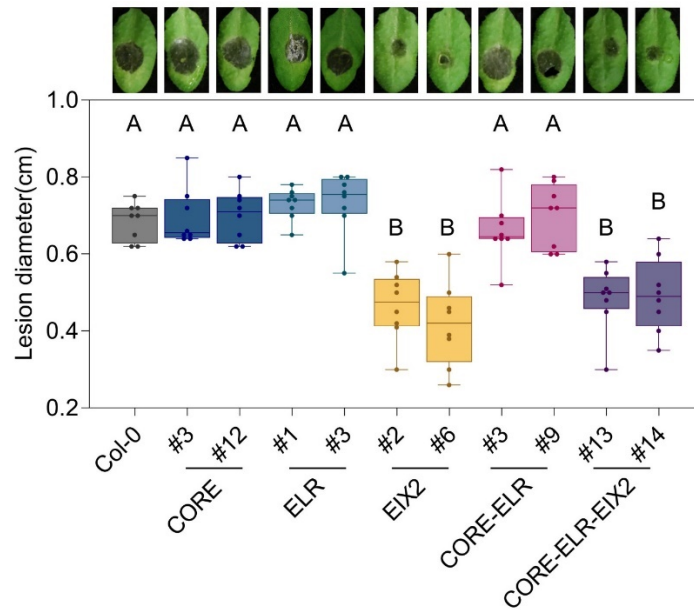


Figure 3.9. EIX2-expressing Arabidopsis plants enhance resistance to fungal pathogen *Botrytis cinerea*.

2×10^6 spores/ml *B. cinerea* were inoculated on Arabidopsis leaves and lesion development was evaluated at 2 dpi. The lesion diameter was measured by ImageJ. Data points are indicated as dots from three independent experiments (n=8). Box plots show the minimum, first quartile, median, third quartile, and maximum lesion diameter. Different letters above the box plot indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$).

3.1.5.3 ELR-expressing lines enhance resistance to *Albugo laibachii*

To investigate whether ELR-expressing transgenic Arabidopsis plants are more resistant to oomycetes, an obligate biotroph oomycete *Albugo laibachii* was chosen for infection assay. The leaves of Col-0 and transgenic lines were sprayed with *A. laibachii* Nc14 zoospore suspension. After 9d inoculation, ELR-, CORE-ELR- and CORE-ELR-EIX2-expressing transgenic lines showed less white blisters on the abaxial side of leaves than that of Col-0 and the transgenic lines without expressing ELR, suggesting that ELR conferred resistance to *A. laibachii* (Figure 3.10).

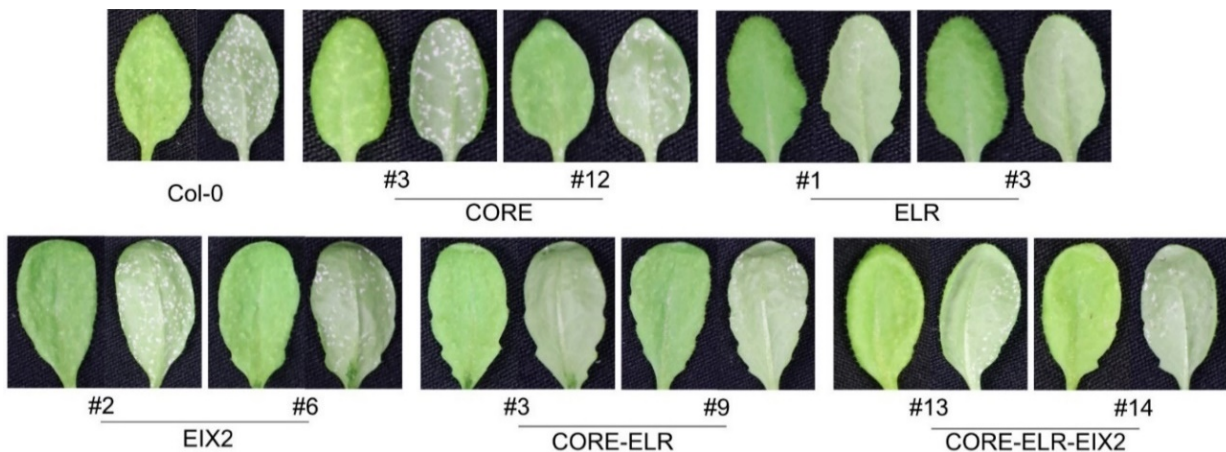


Figure 3.10. ELR-expressing transgenic Arabidopsis plants enhance resistance to oomycete *Albugo laibachii*.

Results

Three to four-week-old plants were sprayed with 1×10^5 spores/ml *A. laibachii* zoospore suspension. Disease symptoms of the adaxial (left) and abaxial (right) sides of the leaves were photographed at 9 dpi.

Collectively, CORE-, ELR-, and EIX2-expressing Arabidopsis plants activate immune responses upon corresponding elicitation, and increase resistance to pathogens *Pst* DC3000, *A. laibachii*, and *B. cinerea*, respectively. Additionally, the stacking of CORE-ELR and CORE-ELR-EIX2 in Arabidopsis confers similar resistance as the single receptor-expressing plants without loss or interference with each other's function. These results suggest that the interfamily transfer of multiple PRRs can be used as a strategy to engineer plants to gain broad-spectrum resistance to pathogens.

3.2 Pyramiding PRR gene expression in *N. benthamiana*

The stacking of CORE-ELR-EIX2 increases broad-spectrum resistance to different pathogen species in Arabidopsis plants as described above, demonstrating that ectopic expression of PRRs which recognize different PAMPs derived from different microbes performs vigorous immune functions. The combination of PERU and RLP23 transferred into potato renders more resistance to *Phytophthora infestans* infection which reveals the additional effects of two oomycete receptors compared with a single receptor during interfamily transformation (Ascurra et al., 2023). To further explore whether stable expression of two bacterial receptors simultaneously can confer robust resistance the tomato RLK SICORE and SIFLS3, and the RLP SIEIX2 were used for pyramiding in one binary vector and transformed into *Nicotiana benthamiana*.

3.2.1 Construction of three receptor gene-cassette *SICORE-SIFLS3-SIEIX2*

PRRs SICORE, SIFLS3, and SIEIX2 coding sequences were retrieved from NCBI and firstly cloned into the corresponding binary vectors respectively which are driven by 35S promoter and C-terminally fused different tags (Figure 3.11a). To generate binary and ternary gene cassettes, 35S-SIFLS3-Myc cassette was amplified by PCR and assembled into the *Apal* site of pB7FWG2.0::SICORE which created pB7FWG2.0::SICORE-eGFP::SIFLS3-Myc (Figure 3.11b), following with UBQ10 promoter and SIEIX2-HA were PCR amplified with templates pRW004-SM and pGWB14::SIEIX2 and placed into *PmeI* site of pB7FWG2.0::SICORE-eGFP::SIFLS3-Myc by Gibson Assembly which produced pB7FWG2.0::SICORE-eGFP::SIFLS3-Myc::UBQ10-SIEIX2-HA (Figure 3.11c).

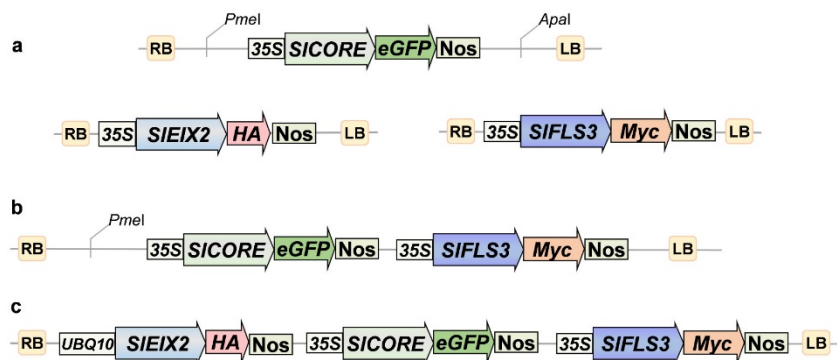


Figure 3.11. Schematic representation of gene cassette construction.

a, One receptor gene cassette. In the single receptor gene expression vectors, SICORE was cloned into pB7FWG2.0 under the control of 35S promoter and with C-terminal eGFP-fusion; SIFLS3 was cloned into pGWB20 under 35S promoter and with C-terminal Myc-fusion; SIEIX2 was cloned into pGWB14 under 35S promoter and with C-terminal HA-fusion by gateway cloning method. **b**, Two receptor genes cassette. After digesting plasmid pB7FWG2.0::SLCORE by *Apal* restriction enzyme, the fragment P35S-SIFLS3-Myc-Tnos amplified using specific primers (Table 2.4) was assembled to linearized pB7FWG2.0::SICORE by Gibson Assembly method. **c**, The three genes cassette was constructed by linearized pB7FWG2.0::SICORE-SIFLS3 using *PmeI* enzyme and two fragments UBQ10 and SIEIX2-HA-Tnos amplified with specific primers utilizing Gibson Assembly to stack these three receptor genes into one vector.

3.2.2 Transient expression of *SICORE-SIFLS3-SIEIX2* cassette in *Nicotiana benthamiana*

To confirm the constructs obtained are functional, the binary vectors pB7FWG2.0::*SICORE*, pGWB20::*SIFLS3*, pGWB20::*SIEIX2*, pB7FWB2.0::*SICORE-SIFLS3*, and pB7FWB2.0::*SICORE-SIFLS3-SIEIX2* (hereafter CORE, FLS3, EIX2, CORE-FLS3, CORE-FLS3-EIX2) were transformed into *A. tumefaciens* strain GV3101 competent cells by electroporation. The agro transformants carrying the corresponding plasmid were applied for transient expression assay in *N. benthamiana*. After agroinfiltration into four to five-week-old *N. benthamiana* leaves, the leaves were treated with 100 nM csp22, 100 nM flgII-28 for ROS burst measurement, or with 200 nM xylanase for ethylene production assay. The results showed that single CORE-expression, and both gene cassettes (CORE-FLS3, CORE-FLS3-EIX2)-expressing lines caused ROS burst upon csp22 treatment (Figure 3.12a). Likewise, the plants expressing FLS3 alone and the plants expressing gene cassettes containing FLS3 (CORE-FLS3, CORE-FLS3-EIX2) triggered ROS burst upon flgII-28 perception (Figure 3.12b). Transient expression of EIX2 and CORE-FLS3-EIX2 also triggered ethylene accumulation upon xylanase treatment (Figure 3.12c). However, untransformed *N. benthamiana* leaves had no response to xylanase treatment. SCP^{Ss} which is recognized by NbRE02 receptor in *N. benthamiana* served as a positive control in the ethylene assay (Yang et al., 2023). To examine the expression levels of these receptors, total proteins were extracted and loaded in SDS-PAGE gels (Figure 3.14b). As the immune activities and protein expression were determined, these gene cassettes can be utilized for stable transformation into *A. thaliana*.

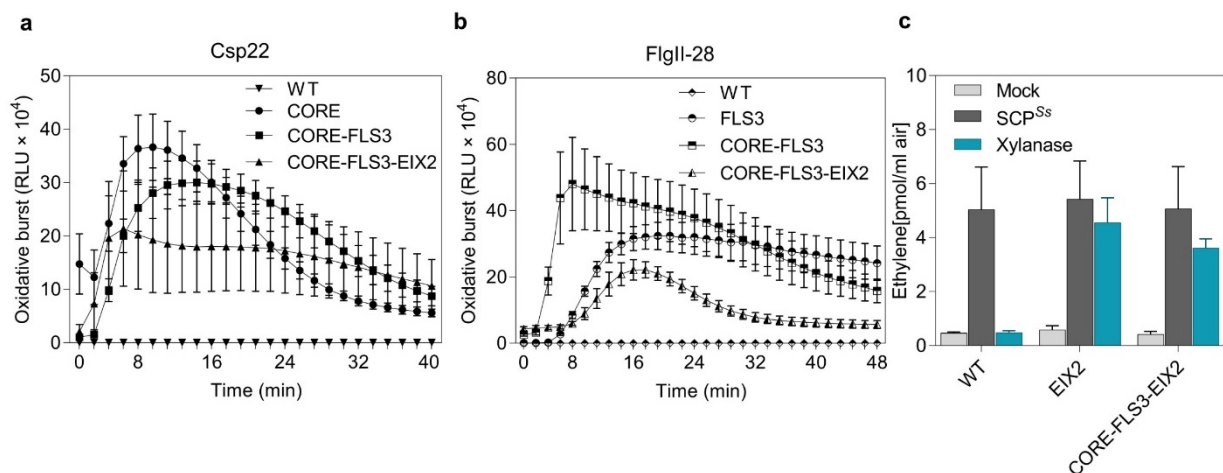


Figure 3.12. Immune outputs in *N. benthamiana* leaves through transient expression of gene cassettes.

a,b, ROS burst in *N. benthamiana* leaf discs transiently transformed CORE, CORE-FLS3, or CORE-FLS3-EIX2 receptors and treated with water (mock), 100 nM csp22 (**a**) or 100 flgII-28 (**b**). Curves show luminol-dependent light emission in 40- or 48-min. Values and error bars show means \pm S.D. of three replicates. **c,** Ethylene production in *N. benthamiana* leaf discs transiently expressed with EIX2,

Results

CORE-FLS3-EIX2 receptors 4 h after treatment with water (mock), 500nM SCP^{Ss} (positive control) or 200 nM xylanase. Bars represent means + S.D. of three replicates.

3.2.3 Stable transformation of CORE-FLS3 in Arabidopsis

To obtain the genetic transformation of CORE-FLS3 in Arabidopsis, the *CORE-FLS3* gene cassette was used for transformation into *A. thaliana* Col-0 background by *Agrobacterium*-mediated transformation. T0 seeds were screened with the Basta antibiotic and selected for the regeneration of T1 plants. Then, putative transformants were confirmed by qPCR and protein levels of FLS3 which were determined by Western blot assay (Figure 3.13a and b). The positive T1 transgenic lines were treated with water (mock), 100 μ M flg22 (positive control), 100 μ M csp22, or 100 μ M flgII-28 for ROS burst measurement in a time course of 40 min. Both the Col-0 and transgenic lines had ROS response to flg22 treatment, however, the CORE-FLS3 transgenic lines only responded to csp22 treatment, not to flgII-28 (Figure 3.13c). These results indicate that the RNA transcription and protein levels of FLS3 were detected, while unlike the ROS burst elicited by csp22, the immune response triggered by flgII-28 was defective. Further research showed that FLS3 protein accumulation was not detectable in FLS3-transformed Arabidopsis plants (Hind et al., 2016). Since FLS3 cannot function properly in Arabidopsis, another model plant, *Nicotiana benthamiana*, was chosen for gene-cassette (containing FLS3) stable transformation and disease resistance evaluation.

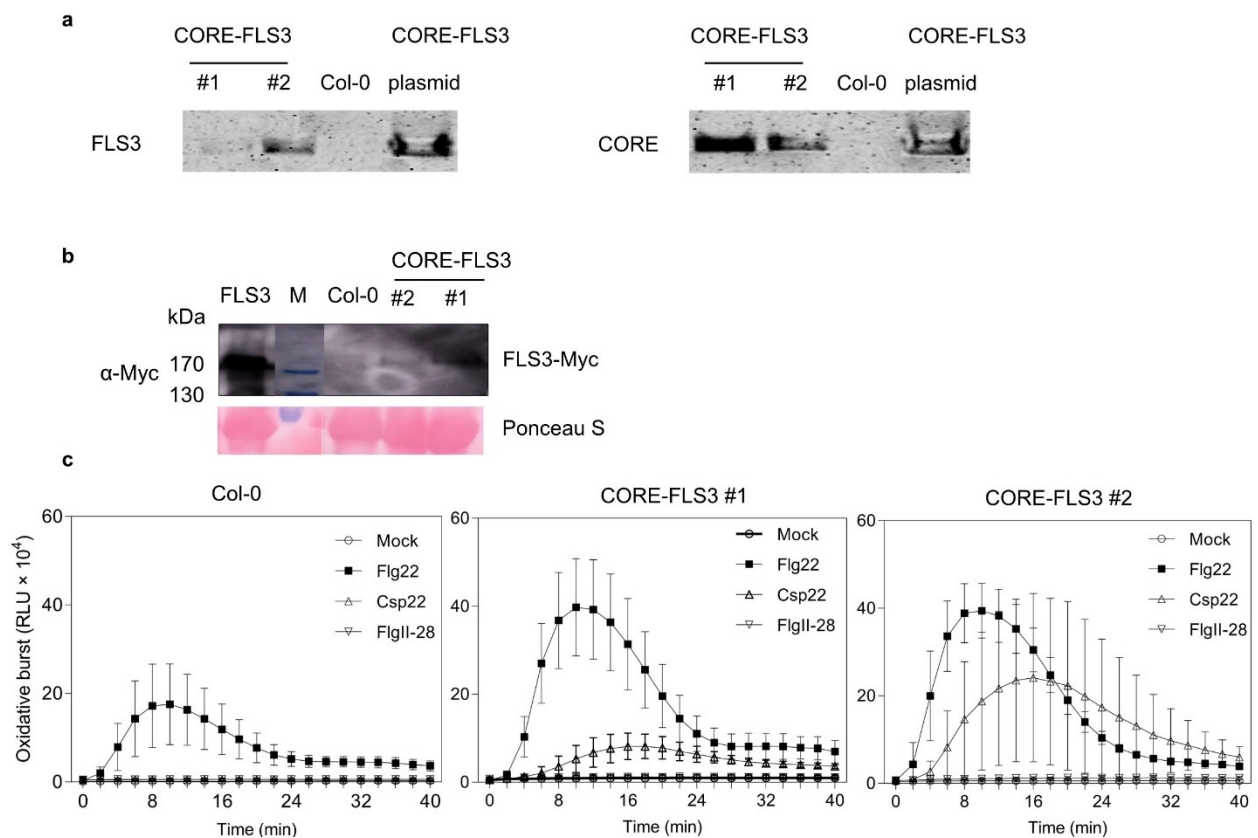


Figure 3.13. Characterization of CORE-FLS3-expressing lines in *A. thaliana*.

a, Agarose gel electrophoresis of qPCR products of CORE and FLS3 target genes. The total RNA was extracted from leaves of T1 generation lines (#1, #2) and Col-0, and cDNAs were synthesized from

Results

RNA. qPCR was performed using Col-0 cDNA (serves as negative control), CORE-FLS3 plasmid (serves as positive control), CORE-FLS3 #1 and #2 cDNA as templates and specific primers. **b**, The protein accumulation of FLS3-Myc in T1 generation leaves was determined by Western blot with Myc antibody. Total protein from Col-0 (right side of the marker, M) and proteins from the transient expression of receptors (left side of the marker) served as negative and positive controls, respectively. **c**, Leaf discs from Col-0 and stable transformed CORE-FLS3 Arabidopsis plants were treated with water (mock, as a negative control), 100 nM flg22 (as a positive control), 100 nM csp22 or 100 nM flgII-28. Curves show luminol-dependent light emission in 40 min. Values and error bars show means \pm S.D. of three replicates.

3.2.4 Stable transformation of single genes and multiple gene cassettes in *N. benthamiana*

After verification of gene expression by transient expression, the receptors were stably transformed in *N. benthamiana* to generate stable transformants by *Agrobacterium*-mediated transformation. T1 generation plants regenerated from T0 explants were transferred from the cultivation medium to soils and screened by genomic DNA PCR analysis and the protein level of each receptor (Figure 3.14a and b). The positive T1 transgenic plants were maintained for seeds and T2 generation transformants were screened by immune responses and selected for infection assay. All T2 transformants showed normal plant growth and seed production, thus two independent transgenic lines from each transformation were chosen for further studies.

Results

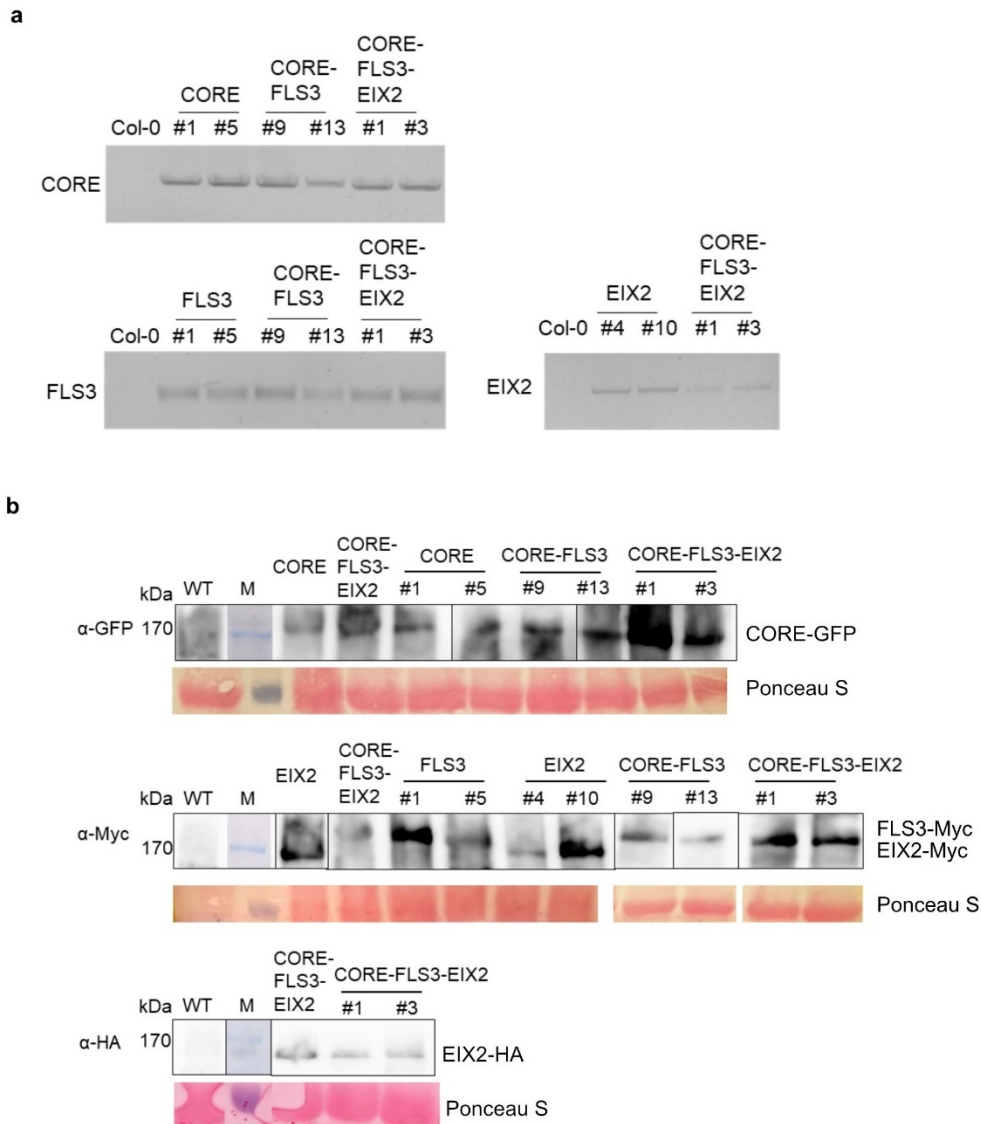


Figure 3.14. Characterization of stable transgenic lines in *N. benthamiana*.

a, PCR amplification of *SICORE*, *SIFLS3*, and *SIEIX2* gene fragments using genomic DNA extracted from leaves of T1 generation. Genomic DNA extracted from wild-type plants serves as a negative control. **b**, The protein accumulation of CORE-GFP, EIX2-Myc (in pGWB20::*SIEIX2*), FLS3-Myc (in pGWB14::*SIFLS3*, pB7FWG2.0::*SICORE-eGFP::SIFLS3-Myc*, and pB7FWG2.0::*SICORE-eGFP::SIFLS3-Myc::UBQ10-SIEIX2-HA*) and EIX2-HA (in pB7FWG2.0::*SICORE-eGFP::SIFLS3-Myc::UBQ10-SIEIX2-HA*) in T1 generation leaves was determined by Western blot with the corresponding antibodies. Total protein from wild type (left side of the marker, M) and proteins from transient expression of receptors (right side of the marker) served as negative and positive control, respectively.

3.2.5 Immune response in *N. benthamiana* transgenic plants

Since immune responses elicited by ligand recognition are typical in pattern-triggered immunity and can limit and have toxic effects on pathogen progression, it is essential that defense responses are detectable in PRR transgenic plants upon ligand treatment to enhance defense and resistance against pathogen infection. In this section, ROS burst, ethylene production, and hypersensitive response assays are performed with the corresponding ligands in T2 transgenic lines.

3.2.5.1 ROS burst in *N. benthamiana* transgenic lines

To determine whether the expression of CORE, FLS3, and EIX2 in *N. benthamiana* responds to csp22, flgII-28, and xylanase, respectively, the selected T2 transgenic lines with these single receptors and gene cassettes as well as wild-type plants were treated with water (mock), 100 nM flg22, 100 nM csp22, 100 nM flgII-28, or 2 μ M xylanase and measured for oxidative burst in a time course of 45 min. Both transgenic lines and wild-type plants released ROS upon flg22 induction, while only CORE-expressing lines showed ROS burst upon csp22 treatment, FLS3-expressing lines upon flgII-28 treatment, and EIX2-expressing lines upon xylanase treatment. Not surprisingly, there was no ROS response in wild-type plants and unmatched receptor expression lines upon ligands treatments (Figure 3.15a). The xylanase, ethylene-inducing xylanase (EIX), which was initially identified to induce ethylene production (Hanania et al., 1999), has recently reported to elicit ROS burst in tomato (Anand et al., 2021). These results demonstrate that EIX2 expression can also confer ROS burst triggered by xylanase in *N. benthamiana* and gene-pyramiding can perform ROS response upon ligand treatment without functional interference.

In CORE-FLS3 transgenic lines, csp22 and flgII-28 can trigger ROS burst respectively, however, whether these two bacterial receptors are able to respond to their ligands at the same time and cause additive output to csp22 and flgII-28 together than the single ligand treatment. In the CORE-FLS3 transgenic lines, the leaf discs were treated with 10 nM csp22, 10nM flgII-28, or 10 nM csp22 + 10 nM flgII-28, both # 9 and # 13 lines have similar ROS production so that the # 9 line was chosen for replicating. The results showed that 10 nM csp22 can trigger ROS burst but weaker, the curve triggered by 10 nM flgII-28 was apparent. Surprisingly 10 nM csp22 + 10 nM flgII-28 can trigger more ROS burst than either sole ligand (Figure 3.15b). These results certify that two bacterial receptor CORE-FLS3 expression can lead to more immune output upon simultaneous PAMPs treatment.

Results

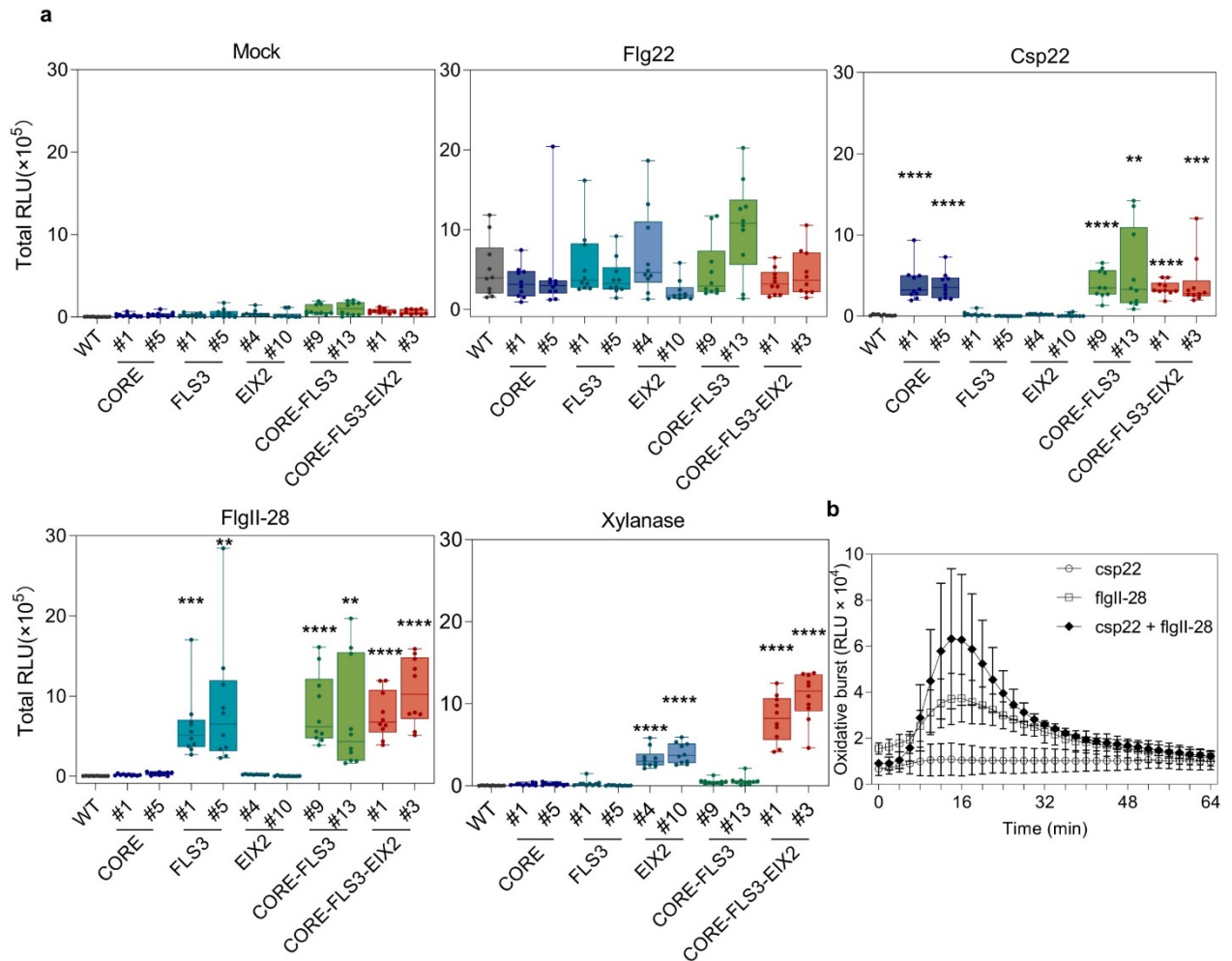


Figure 3.15. ROS burst in *N. benthamiana* transgenic plants.

a, Leaf discs cut from wild type and transgenic lines were treated with water (mock, as a negative control), 100 nM flg22 (as a positive control), 100 nM csp22, 100 nM flgII-28, or 2 μ M xylanase. The total oxidative burst was summed up in 45 min. Data points are indicated as dots from three independent experiments ($n=10$). Box plots show the minimum, first quartile, median, third quartile, and a maximum of total ROS burst. Asterisks indicate significant differences to wild type by two-tailed Student's *t*-test (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). **b**, The CORE-FLS3 transgenic line # 9 was treated with 10 nM csp22, 10 nM flgII-28, or 10 nM csp22 + 10 nM flgII-28. Curves show luminol-dependent light emission integrated in 64 min. Values and error bars show means \pm S.D. of three replicates.

3.2.5.2 Ethylene accumulation in *N. benthamiana* transgenic plants

To determine the ethylene accumulation induced by csp22, flgII-28 and xylanase in *N. benthamiana* plants stably transformed with PRRs, leaf discs were treated with water (mock), 0.5 μ M SCP^{Ss} (as positive control), 2 μ M csp22, 2 μ M flgII-28 or 200 nM xylanase. As the results showed, all the plants responded to SCP^{Ss}, while only CORE-expressing, FLS3-expressing and EIX2-expressing lines were responsive to csp22, flgII-28 and xylanase treatment, respectively. While wild-type plants and transformants with unmatched receptors had no ethylene response to csp22, flgII-28 and xylanase elicitation (Figure 3.16a). These results indicate that expression of these PRRs in *N. benthamiana* are able to produce ethylene specifically upon their cognate PAMPs treatment.

Results

Since ROS burst activated by *csp22* + *flgII-28* together is greater than single ligand treatment, we tested whether ethylene accumulation is also enhanced. CORE-FLS3 transgenic line #9 was treated with 1.5 μM *csp22*, 1.5 μM *flgII-28*, or 1.5 μM *csp22* + 1.5 μM *flgII-28* to verify this hypothesis. Both #9 and #13 lines have similar ethylene production so the #9 line was chosen for replication. As the results showed, two ligands added together indeed induced more ethylene production than single ligand (Figure 3.16b). These results suggest that CORE-FLS3 expression plants can recognize two cognate ligands synchronously and lead to much more immune outputs when treated with both ligands compared to one ligand.

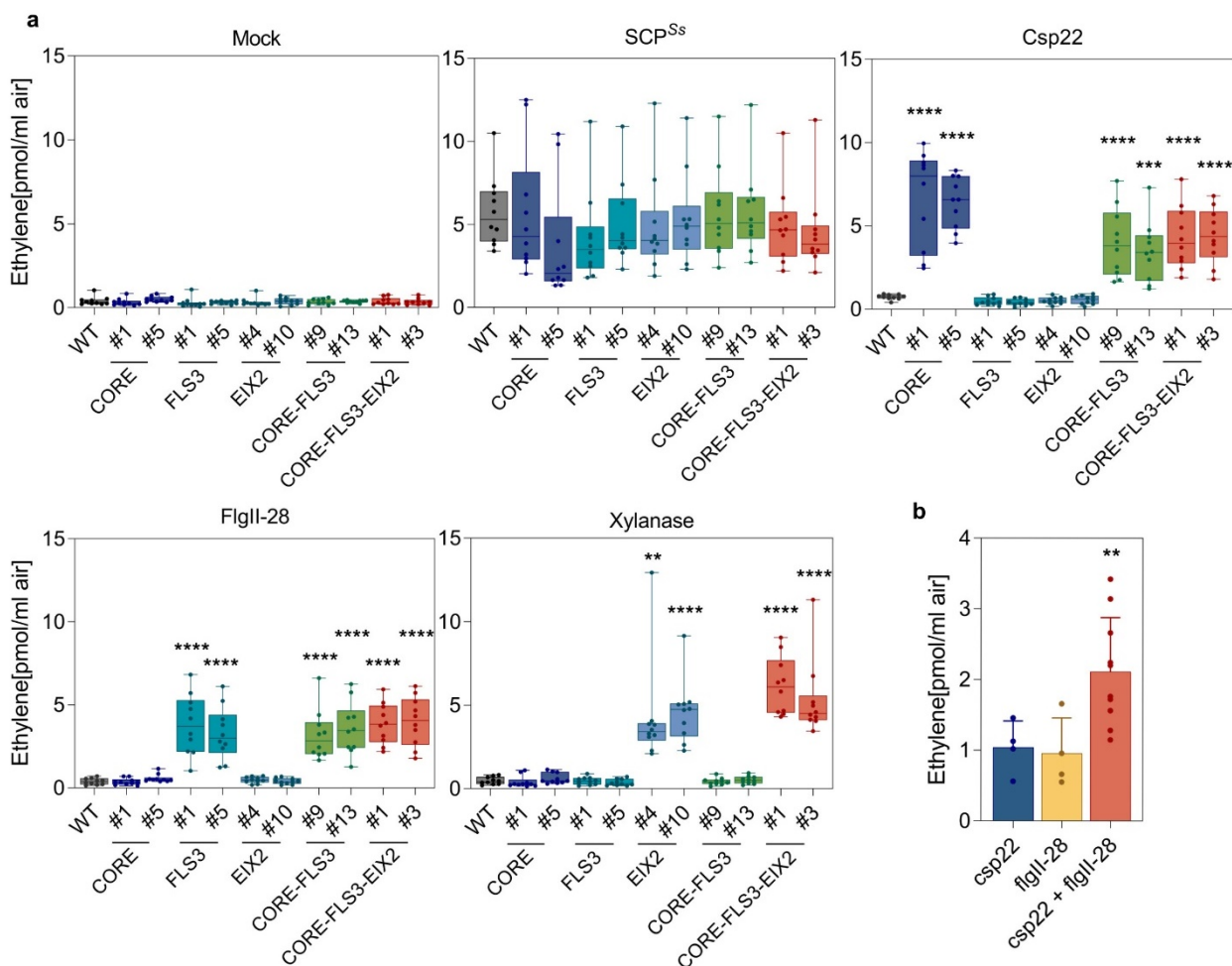


Figure 3.16. Ethylene production in transgenic *N. benthamiana*.

a, Leaf discs cut from wild type and transgenic lines were incubated with water (mock), 0.5 μM SCP^{Ss} (as a positive control), 2 μM *csp22*, 2 μM *flgII-28*, or 200 nM xylanase. Data points are indicated as dots from three independent experiments ($n=10$). Box plots show the minimum, first quartile, median, third quartile, and a maximum of ethylene production. Asterisks represent significant differences to wild type by two-tailed Student's *t*-test (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). **b**, The CORE-FLS3 transgenic line #9 was treated with 1.5 μM *csp22*, 1.5 μM *flgII-28*, or 1.5 μM *csp22* + 1.5 μM *flgII-28* to measure oxidative burst. Bars represent means + S.D. of three replicates ($n=4$). Asterisks show significant differences to treatment of *csp22*, *flgII-28* individually following one-way ANOVA (** $P \leq 0.01$).

3.2.5.3 Hypersensitive Response in EIX2-expressing transgenic *N. benthamiana*

Leaves of tobacco *Nicotiana tabacum* cv Samsun develop cell death after injection with xylanase, whereas infiltration of xylanase in *N. benthamiana* wild type results in no reaction (Ron & Avni, 2004). To determine whether the expression of EIX2 in *N. benthamiana* is able to execute cell death after xylanase injection, all the transgenic lines and wild-type plants were infiltrated with 20 μ M xylanase. After 7 d infiltration, EIX2-expressing lines showed cell death upon the infiltration areas (marked in circles), while wide-type plants and other transgenic plants without EIX2 expression exhibited no visible response to xylanase (Figure 3.17). These observations indicate that the recognition of xylanase by EIX2 leads to cell death in EIX2- and CORE-FLS3-EIX2-expressing *N. benthamiana*.

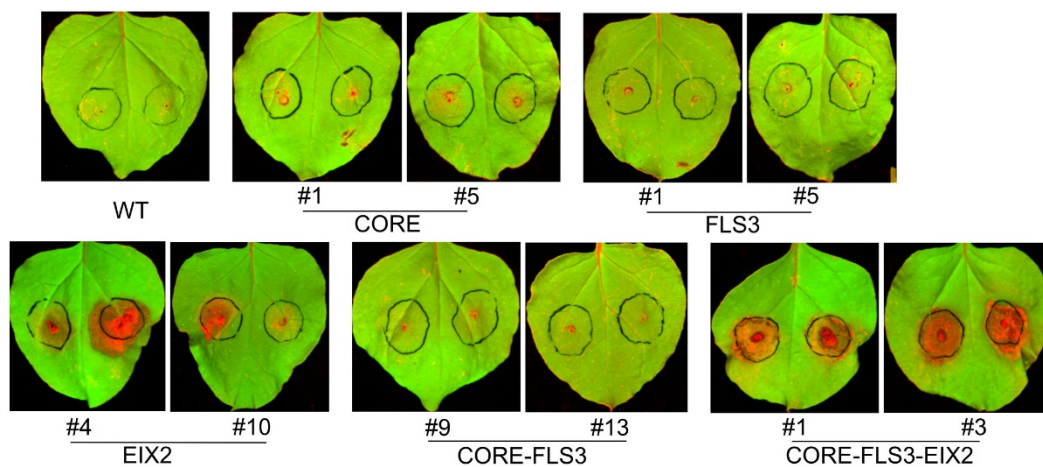


Figure 3.17. Cell death in EIX2-expressing *N. benthamiana*.

Leaves were infiltrated with 20 μ M xylanase and visualized under the UV light using an Amersham ImageQuant 800 and an integrated Cy5 filter at 7 d post infiltration.

3.2.6 Expression of receptors and pyramiding receptors leads to resistance to pathogens

Stable transformation of stacking PERU and RLP23 in potato has been successfully observed to increase resistance to *P. infestans* compared with either single receptor transformation (Ascurra et al., 2023). The immune outputs, ROS burst and ethylene production, are additionally elicited by transferring stacked bacterial receptors CORE-FLS3 when triggered with *csp22* and *flgII-28* together. We next tested whether CORE-FLS3 expression provides additional resistance to bacteria as compared to single bacterial receptor transformation.

3.2.6.1 Expression of CORE-FLS3 confers resistance similar to that of single receptor expression to *Pseudomonas syringae* pv. *tabaci* (*Pstab*) infection

To discover whether the stable transformation of CORE and FLS3 in *N. benthamiana* was able to increase resistance to bacteria, 4-week-old transgenic and wild-type plants were infiltrated with *Pstab* in the marked circle site of leaves. After 4 d infiltration, bacteria

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proliferation was determined by colony quantities grown on medium and analyzed using statistics. The results showed that bacterial growth was limited in CORE-expressing and FLS3-expressing lines compared to wild-type and EIX2 transgenic lines (Figure 3.18a). However, stacking of CORE-FLS3 lines performed similar bacteria growth instead of less growth compared to the individual CORE- and FLS3-expressing plants. Cell death caused by *Pstab* colonization visualized obvious limitation of bacterial expansion in the injection circle of CORE-, FLS3-, and CORE-FLS3-expressing plants, while bacterial expansion was spread much over injection areas in EIX2-expressing lines and wild-type plants (Figure 3.18b).

Interestingly, CORE-FLS3-EIX2 expressing *N. benthamiana* exhibited significantly less bacterial growth than lines expressing CORE, FLS3, and CORE-FLS3 upon *Pstab* infection. Cell death in CORE-FLS3-EIX2 transgenic plant leaves showed that not only bacteria growth was restricted in the injection circle, but also quantity of colonization was apparently less than in other tested plant leaves. These results imply that stacking two receptors recognizing bacterial patterns cannot confer more resistance than a single receptor. On the contrary, expression of CORE-FLS3-EIX2 showed additional resistance to *Pstab*, indicating that the fungal receptor EIX2 possesses potential function to promote CORE-FLS3 resistance.

Results

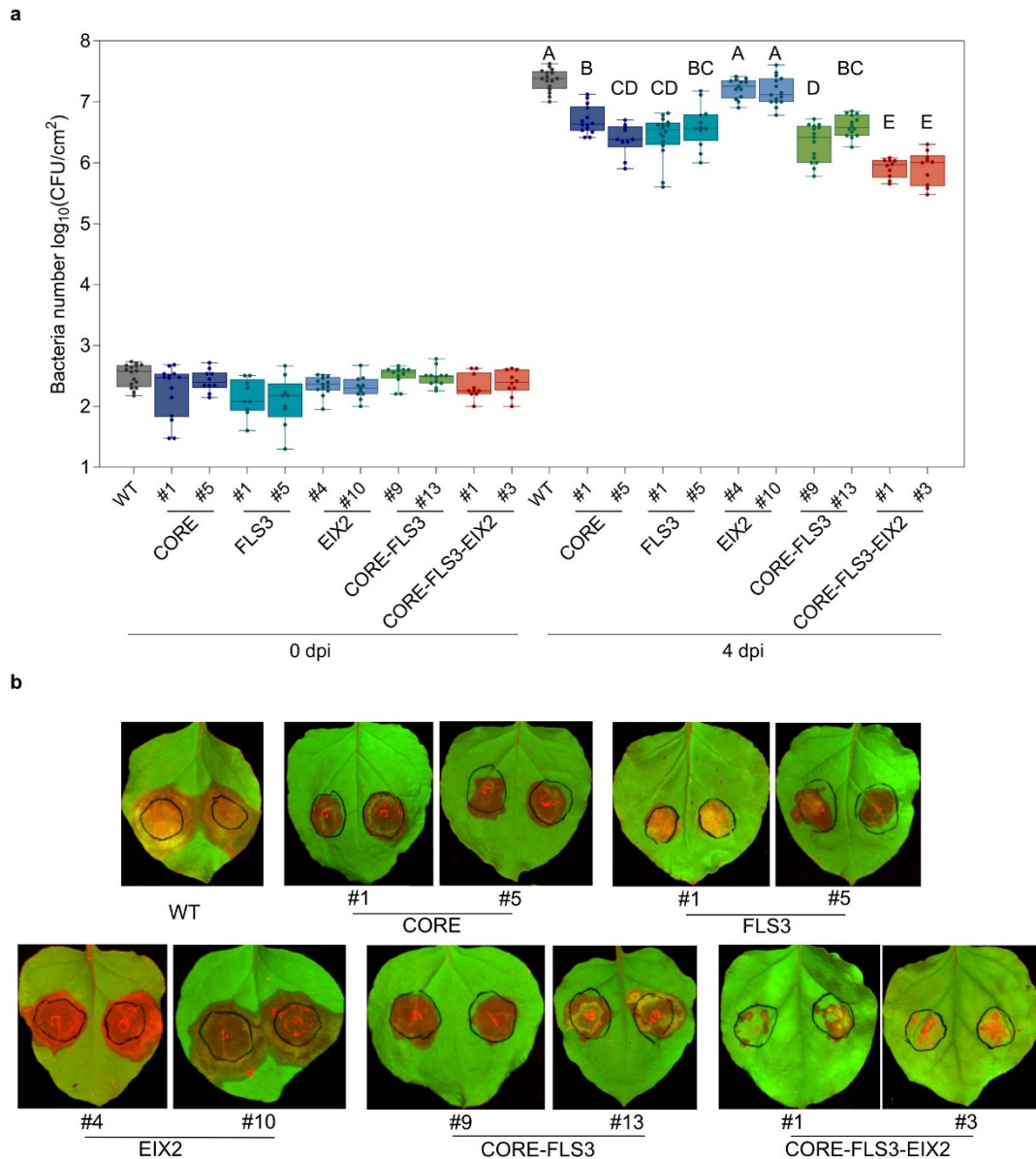


Figure 3.18. CORE- and FLS3-expressing *N. benthamiana* plants increase resistance to bacterial pathogen *Pstab*.

a. *N. benthamiana* wild type and transgenic lines expressing CORE, FLS3, EIX2, CORE-FLS3, and CORE-FLS3-EIX2 were infiltrated with 1×10^4 cfu/ml *Pstab*. Bacteria quantified in extracts of leaves at 0 and 4 d post-infiltration. Data points are indicated as dots from three independent experiments (n=10). Box plots show the minimum, first quartile, median, third quartile, and a maximum of log cfu/cm² leaf tissues. Different letters above the box blot at 4 dpi indicate statistically differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$). **b.** Cell death was photographed at 4 d post-infiltration under the UV light using an Amersham ImageQuant 800 and an integrated Cy5 filter.

3.2.6.2 Expression of EIX2 in *N. benthamiana* enhances resistance against *Botrytis cinerea*

To investigate whether expression of EIX2 renders *N. benthamiana* more resistant to fungus *B. cinerea*, 4-week-old *N. benthamiana* wild-type and transgenic plants were inoculated with *B. cinerea* B05.10 spores. After 2 day-inoculation, the lesion sizes were significantly smaller in EIX2- and CORE-FLS3-EIX2-expressing lines, than those in wild-type plants and

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transgenic lines without EIX2 (Figure 3.19). However, entire transgenic lines and wild-type plants would be colonized and destroyed by *B. cinerea*, even though remission of infection in EIX2-expressing plants cannot be continuous either, which was observed after 4-day-inoculation. These results suggest that expression of EIX2 and CORE-FLS3-EIX2 successfully confer early infection stage resistance to the devastating fungus *B. cinerea*.

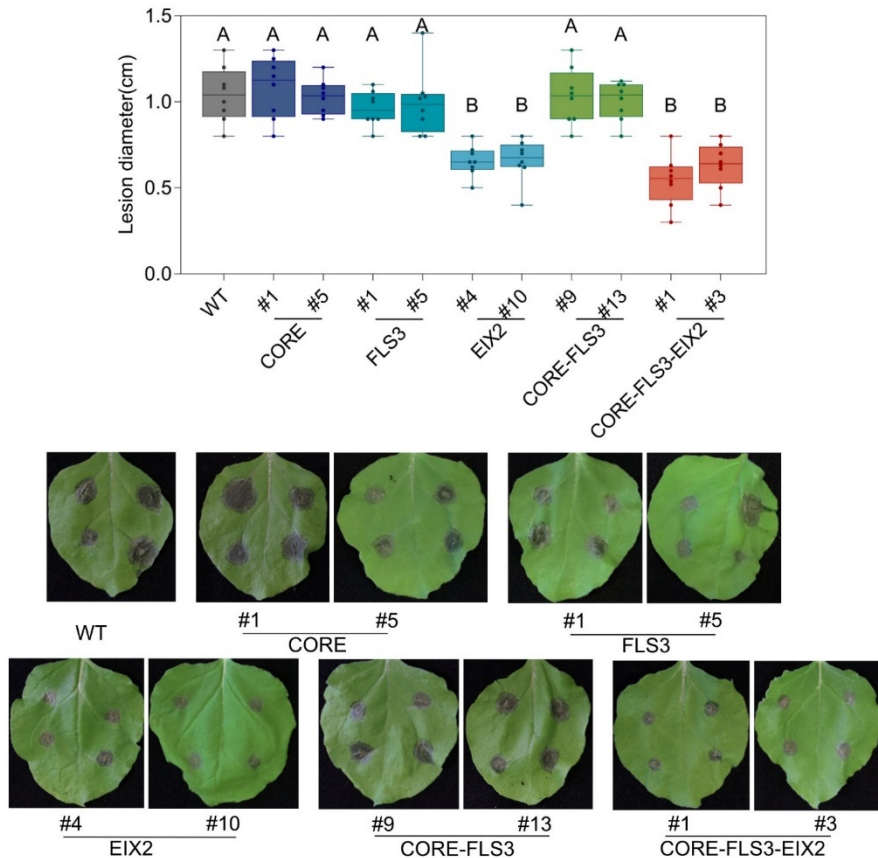


Figure 3.19. EIX2 transgenic *N. benthamiana* plants increase resistance to fungal pathogen *B. cinerea*.

The transgenic and wild-type plants were inoculated with $2 \mu\text{l}$ 1×10^4 spores/ml *B. cinerea* B05.10 spores on the two sides of midrib avoiding veins. Lesion development was evaluated at 2 dpi and lesion diameter was measured by ImageJ. Data points are indicated as dots from three independent experiments ($n=8$). Box plots show the minimum, first quartile, median, third quartile, and a maximum lesion diameter. Different letters above the box blot indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$). Disease symptoms were photographed at 2 dpi.

3.2.6.3 Expression of CORE, FLS3, and EIX2 confers no resistance to *Phytophthora capsici*

P. capsici is a typical oomycete plant pathogen that infects many Solanaceous crops and leads to root rot and brown lesion. To evaluate whether expression of CORE, FLS3, and EIX2 in *N. benthamiana* results in resistance to oomycete, transgenic lines and wild-type plants leaves were inoculated with fresh *P. capsici* hyphae plugs. After 2 day-inoculation, lesion sizes were analyzed and showed that there were no significant differences among transgenic and wild-type plants in the infection process, no matter in the early or late stage

Results

(Figure 3.20). To further assess the resistance to oomycetes, all plants were infected with another oomycete *Phytophthora infestans*. The results were the same as with *P. capsici* infection (Supplementary Figure 7.3). Since CORE and FLS3 are receptors recognizing bacterial PAMPs, and EIX2 is a receptor recognizing a fungal PAMP, it's reasonable that these transgenic plants confer no resistance to oomycete infection. Thus CORE, FLS3, and EIX2 can only confer resistance to bacterial and fungal pathogens.

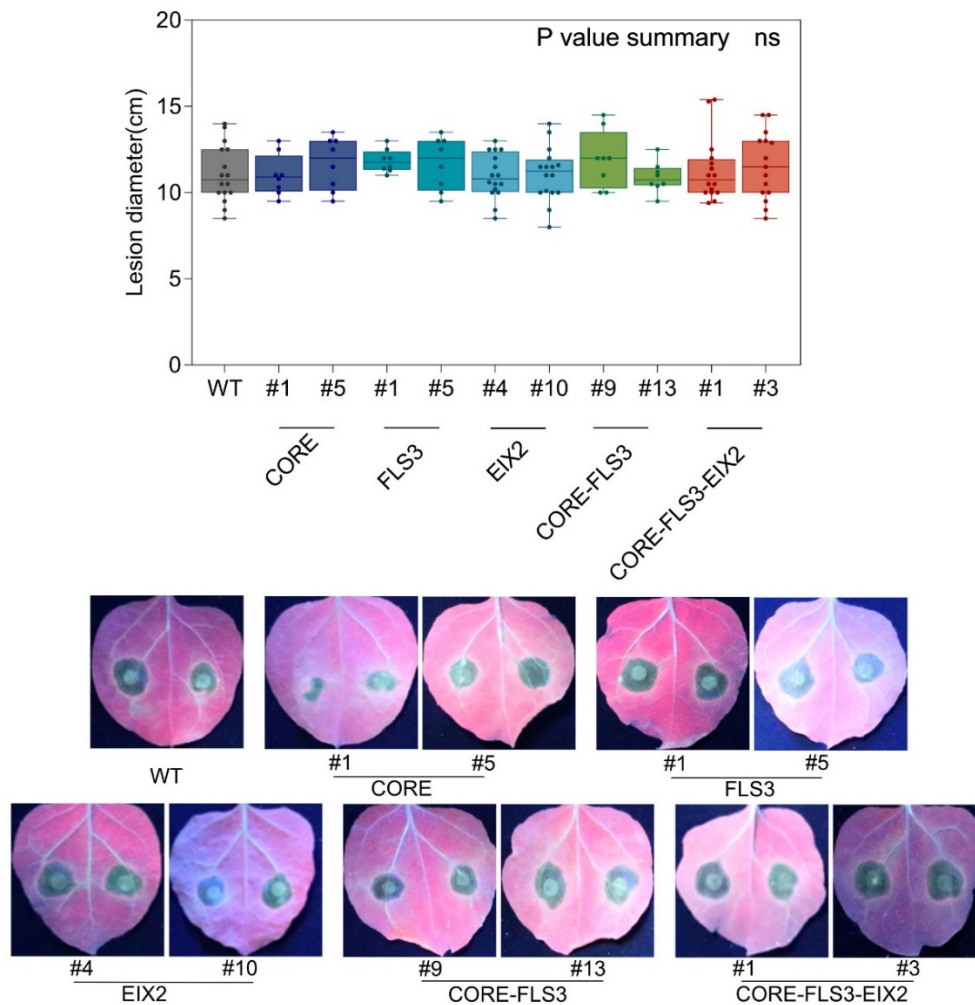


Figure 3.20. CORE-, FLS3-, and EIX2-expressing lines have no resistance to *P. capsici*.

The transgenic and wild-type plants were inoculated with 0.5 cm² *P. capsici* hyphae plugs on the two sides of midrib. Lesion development was evaluated under UV light, and lesion diameter was measured by ImageJ at 2 dpi. Data points are indicated as dots from three independent experiments (n=8). Box plots show the minimum, first quartile, median, third quartile, and a maximum lesion diameter. Letter ns above the box blot indicates statistically non-significant differences among homogenous groups following Duncan's one-way ANOVA (ns $P > 0.05$).

3.2.7 Biomass and phenotype of transformation progeny in *N. benthamiana*

Since immunity often comes with yield penalties, it is crucial to monitor the developing process and growth transformants (Y. Wang et al., 2016). The T2 generation transformants were recorded in the following index to evaluate whether there were some defects in growth: plant wet and dry weight of aerial part, plant seeds weight, and germination of T2 generation

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seeds. All the plants were grown under the same conditions and the plant seeds were collected from the same number of seed pods. The results showed that all the wet and dry weights of transgenic lines were similar to that of the wild-type plant (Figure 3.21a). In addition, the transgenic plants produced a similar amount of seeds as to the wild-type plant (Figure 3.21b). Moreover, the T2 seeds germinated without deformation or non-germinated (Figure 3.21c). These results indicate that *N. benthamiana* plants expressing these receptors have no growth penalties.

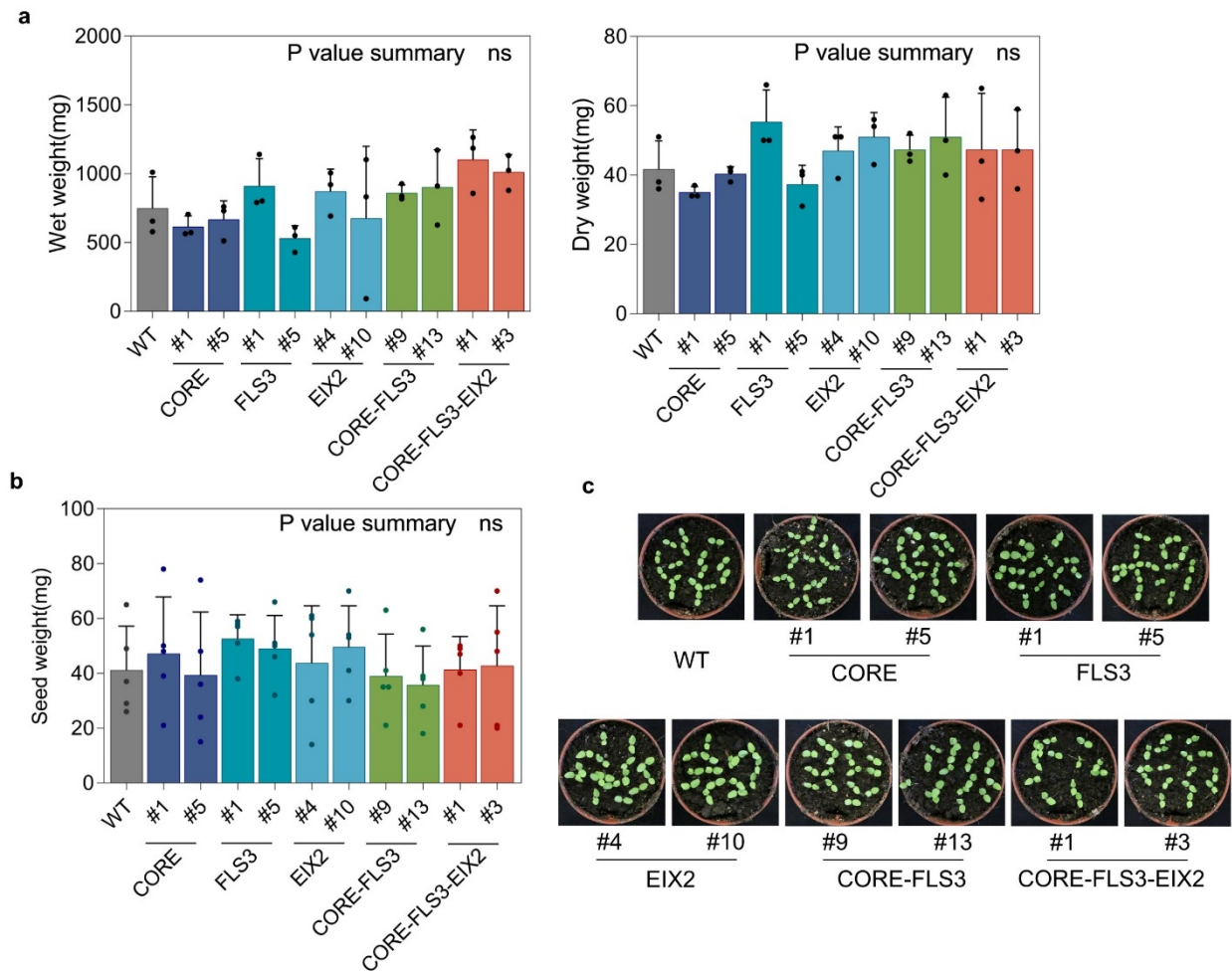


Figure 3.21. Biomass and phenotype of PRRs transformants in *N. benthamiana*.

a, Wet and dry weight measurements on transgenic plants transformed with CORE, FLS2, EIX2, CORE-FLS3, CORE-FLS3-EIX2. Aerial parts of four-week-old plants were weighed wet weight, and dry weight by dehydration in the oven at 100°C for 20 min, then 70°C for 12 h. Bars represent means +S.D. of three replicates. **b**, Seed weight was determined by the weight of seeds derived from three pods of each transgenic line. Bars represent means +S.D. of three replicates. Letters ns indicate non-significant differences among homogenous groups analyzed by Duncan's one-way ANOVA (ns $P > 0.05$). **c**, Representative germination of seeds from wild type and T2 transgenic lines were photographed one week after sowing in soil.

In sum, the stable transformation of PRRs CORE, FLS3, EIX2, and gene cassettes CORE-FLS3, CORE-FLS3-EIX2 mediates defense response and mounts immune resistance to pathogens in *N. benthamiana*. Acting as bacterial receptors, CORE-FLS3 expressing lines

Results

confers synergistic ROS burst and ethylene accumulation by both *csp22* and *flgII-28* elicitation, whereas surprisingly cannot provide additional resistance to *Pstab* infection. Unanticipatedly, stable expression of CORE-FLS3-EIX2 inhibits bacterial growth and renders transgenic plants more resistant to *Pstab* than single or double bacteria receptor transformants. The ectopic expression of receptor EIX2 in *N. benthamiana* provides resistance to *B. cinerea*. As for no resistance to Phytophthora, it's logical since there is no oomycete receptor selected and transferred. This explains why CORE, FLS3, and EIX2 have no positive effect against oomycete infection and shows specificity of the PRRs used for their cognate ligands also upon ectopic expression.

3.3 Mutation of PRRs/co-receptors and evaluation of resistance in Arabidopsis

In PTI, PRRs and co-receptors are implemented for reinforcing immune resistance by perceiving PAMPs derived from pathogens and controlling signaling transduction accompanied with downstream immune response cascade. As so far, the identified and well-characterized bacterial PAMP-recognizing receptors in Arabidopsis comprise LRR-RK FLS2, EFR, XPS, LRR-RP RLP1, RLP32, LysM-RP Lym1/3 and LecRK LORE, fungal-PAMP recognizing receptors LRR-RP RLP30, RLP42, LysM-RK LYK2/4/5, LRR-RK MIK2 and RK WAK1/2, and co-receptors SOBIR1 functioning with LRR-RPs and CERK1 with LYKs and LYMs, are implicated in PTI and pathogen recognition. Often, these single receptor mutants are more susceptible to pathogens (Zipfel et al., 2006; Zipfel et al., 2004). It is unclear whether higher-order mutants (knockouts of more than one receptor) are even more susceptible to pathogens. Therefore, higher order mutants were generated and their susceptibilities to different pathogens were studied in this chapter.

3.3.1 Determination of quintuple mutant *efr fls2 cerk1 lym3 sobir1*

To obtain the quintuple mutant *efr fls2 cerk1 lym3 sobir1* in *A. thaliana*, *efr fls2 cerk1 lym3* mutant was set as the female parent to reduce the possible ratio of heterozygous progeny, and *efr fls2 sobir1* mutant was the male parent for crossing. After pollination of *efr fls2 cerk1 lym3* stigmas with *efr fls2 sobir1* pollen, F1 seeds were obtained in which the genotype should be homozygous *efr fls2* and heterozygous *CERK1cerk1 LYM3lym3 SOBIR1sobir1*. Allelic segregation occurred in F2 progeny by self-pollination in which the homozygote ratio should be 1/64. Therefore, 288 F2 progenies were screened by ethylene assay upon nlp20 treatment and genotyped by PCR with two pairs of CERK1 primers (Supplementary Figure 7.4), 12 plants showing no ethylene production (homozygous *sobir1sobir1*) and no bands upon genotyping were subsequently genotyped by PCR with CERK1 and LYM3 specific primers (Figure 3.22a and b) and phenotyped by measuring ROS burst upon chitin treatment (Figure 3.22c). Eventually, seven lines (# 6-2, # 6-13, # 6-26, # 15-72, #18-33, # 18-58, # 18-78, # 18-99) showed no PCR product and no ROS burst, and thus were identified as homozygous quintuple mutant *efr fls2 cerk1 lym3 sobir1*. These lines are from 3 independent crosses and are kept for seed collection. The lines # 6-2, # 15-72, # 18-78 were chosen for pathogen infection in F3 progeny.

Results

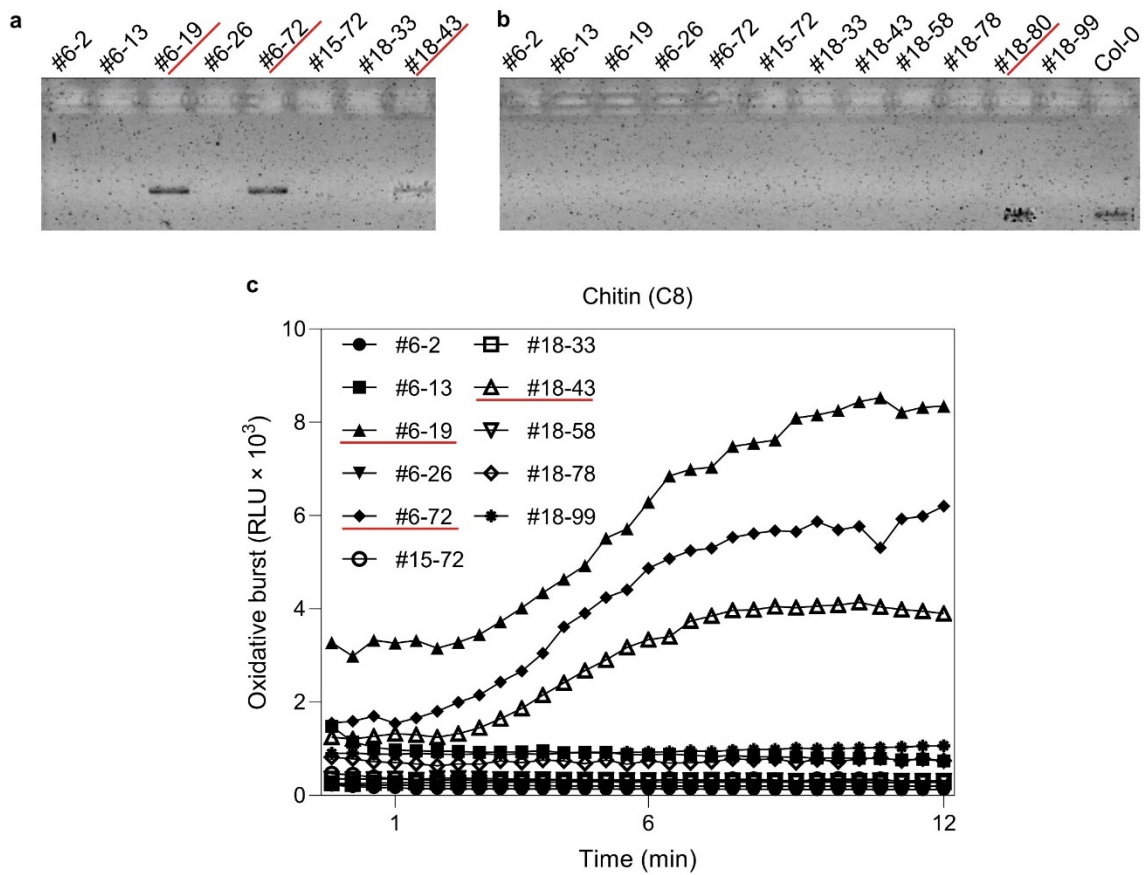


Figure 3.22. Identification of quintuple mutant *efr fls2 cerk1 lym3 sobir1* in *A. thaliana*.

a, b, The F2 generations without ethylene response to *nlp20* were conducted with genotyping. Genomic DNA extracted from lines and Col-0 (as negative control) were amplified with **(a)**CERK1 and **(b)**LYM3 primers. The lines marked with red underlines were heterozygotes. The PCR gel images were adjusted by ImageJ to brighten the weak bands. **c,** The F2 lines were treated with 1 μ M chitin (C8, a polymer consisting of eight N-acetylglucosamine units) to measure ROS burst. The lines without ROS response were homozygotes. The lines marked with red underlines were heterozygotes.

3.3.2 Quintuple mutants are susceptible to *P. syringae* pv. *tomato* (*Pst*) DC3000 infection

The acquisition of mutant *efr fls2 cerk1 lym3 sobir1* indicated that EFR, FLS2, CERK1, LYM3, and all SOBIR1-dependent RLPs like RLP1, RLP32, RLP30, RLP42, RLP30, RLP23 as well as all LYKs and LYMs CERK1-dependent like LYM1/3 and LYK2/4/5 were theoretically abolished in immunity function. To examine whether the quintuple mutant is susceptible to bacterial infection overwhelmingly, double mutants *efr fls2*, triple mutants *efr fls2 sobir1*, *efr fls2 cerk1*, quadruple mutants *efr fls2 cerk1 lym3*, quintuple *efr fls2 cerk1 lym3 sobir1* and Col-0 were sprayed with *Pst* DC3000. The results showed that bacterial growth in all mutants was significantly increased after 2 d inoculation compared to Col-0 (Figure 3.23). However, the three quintuple mutant lines # 6-2, # 15-72, # 18-78 showed no significant difference compared to the double, triple, and quadruple mutants referring to vulnerability in *Pst* DC3000 infection, even though quintuple mutants lost most of bacterial receptors in PTI. The results show that CERK1, LYM3 and SOBIR1-dependent RLPs do not have as essential effects on *Pst* DC3000 resistance as EFR and FLS2 in Arabidopsis.

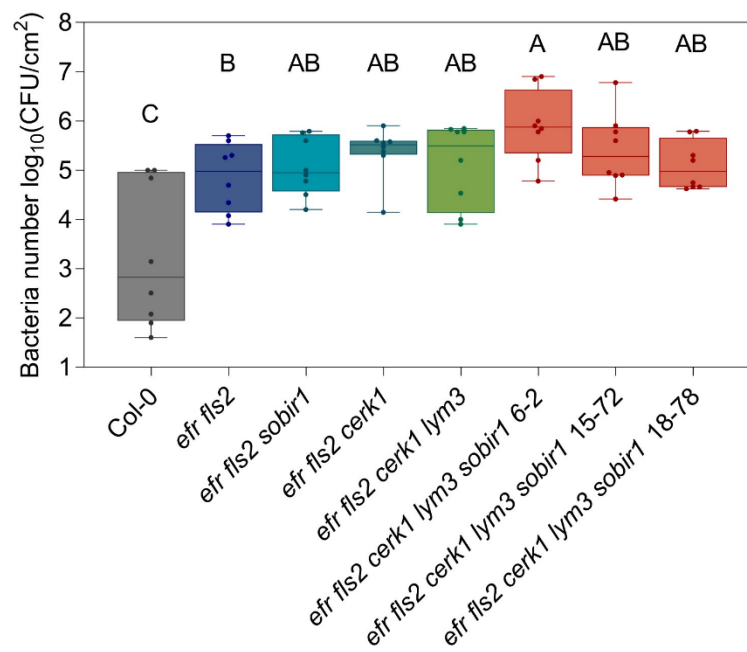


Figure 3.23. Quintuple mutants are susceptible to *Pst* DC3000 infection.

Col-0 and mutants were sprayed with 1×10^7 cfu/ml *Pst* DC3000. Bacterial growth numbers were quantified in extracts of leaves at 2 d post-inoculation. Data points are indicated as dots from three independent experiments (n=8). Box plots show the minimum, first quartile, median, third quartile, and a maximum of \log_{10} cfu/cm² leaf tissues. Different letters above the box blot indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$).

3.3.3 Quintuple mutants are susceptible to *Botrytis cinerea* infection

Since the quintuple mutants not only abrogate bacterial PRRs but also impact fungal PRRs, whether the resistance to fungal infection is decreased in quintuple mutant lines is investigated. Double mutants *efr fls2*, triple mutants *efr fls2 sobir1*, *efr fls2 cerk1*, quadruple

Results

mutants *efr fls2 cerk1 lym3*, quintuple *efr fls2 cerk1 lym3 sobir1* and Col-0 were inoculated with *B. cinerea* spores. After 2 day-inoculation, the quadruple mutant *efr fls2 cerk1 lym3* and quintuple mutants *efr fls2 cerk1 lym3 sobir1* plants showed significantly larger lesion in comparison with double mutant *efr fls2*, triple mutants *efr fls2 sobir1* and *efr fls2 cerk1* and Col-0 (Figure 3.24). The results show that these receptors are fundamental for resistance so that quintuple mutants are more assailable by *B. cinerea*.

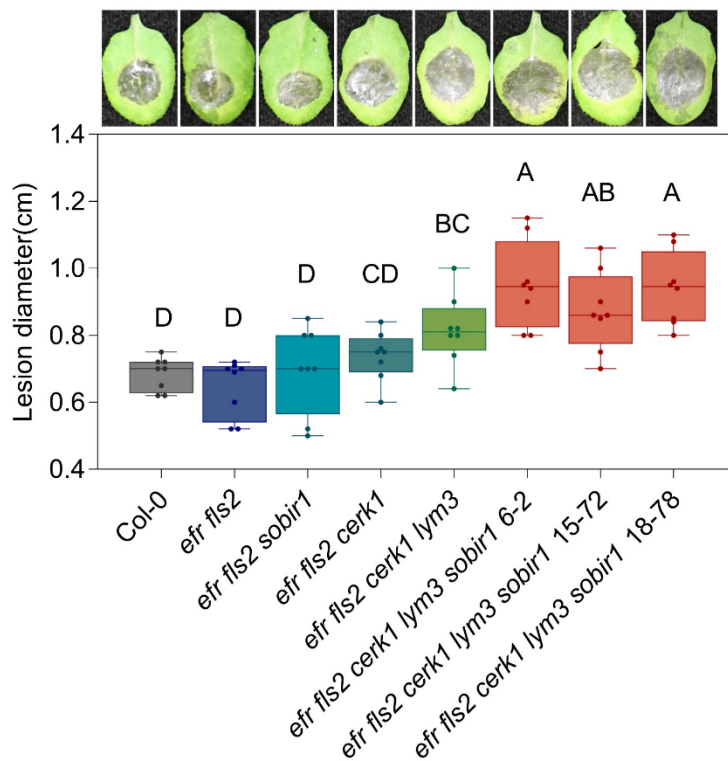


Figure 3.24. Quintuple mutants are susceptible to *Botrytis cinerea*.

2×10^6 spores/ml *B. cinerea* were inoculated on leaves of Col-0 and mutants. Lesion development was evaluated at 2 dpi and lesion diameter was measured by ImageJ. Data points are indicated as dots from three independent experiments ($n=8$). Box plots show the minimum, first quartile, median, third quartile, and a maximum lesion diameter. Different letters above the box plot indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$).

In conclusion, the quintuple mutant *efr fls2 cerk1 lym3 sobir1* eliminating most discovered PRRs functions are subject to *Pst* DC3000 and *B. cinerea* infection, while quintuple mutants show similar succumbing to double, triple mutants in bacterial invasion. Differing from hardly varied susceptibility in bacterial infection, quintuple mutants exhibit significant surrender compared to double and triple mutants upon *B. cinerea* inoculation.

3.4 Arabidopsis PRR expression in tomato and potato

Interfamily transfer of EFR from *A. thaliana* into crop plants confers response to bacterial PAMP elf18 and resistance, demonstrating that transferring PRRs from model plants to crops is an effective way to engineer crop resistance against disease (Lacombe et al., 2010). To investigate whether the introduction of EFR, RLP23, and RLP42 from Arabidopsis to tomato or potato can confer ligand recognition and immune response, the transgenic tomato plants expressing EFR, RLP23, and RLP42 as well as transgenic potato lines expressing EFR were generated. The binary plasmids pK7FWG2.0::*EFR* encoding C-terminally eGFP-tagged EFR, pGWB14::*RLP23* encoding C-terminally HA-tagged RLP23, and pLOC6::*RLP42* encoding C-terminally eGFP-tagged RLP42 were separately transferred to *Solanum lycopersicum* m82 by *Agrobacterium*-mediated transformation, the binary plasmid pK7FWG2.0::*EFR* was transferred to *Solanum tuberosum* Désirée. T0 transformants were selected by tissue culture with corresponding antibiotics and the tomato T1 plants regenerated from T0 plants were screened by genomic DNA PCR analysis (Figure 3.25a).

To test whether the expression of RLP23, RLP42, and EFR confers ethylene production upon corresponding PAMPs treatment, T1 plant leaf discs were treated with water (mock), 5 μ M flg22 (as positive control), 5 μ M nlp20, 5 μ M pg13, or 1 μ M elf18. As the results showed, RLP23-expressing tomato lines were responsive to nlp20, RLP42-expressing tomato lines were responsive to pg13 (Figure 3.25b), EFR-expressing tomato and potato plants were responsive to elf18 (Figure 3.25c). While wild-type plants had no ethylene response to nlp20, pg13, and elf18 elicitation. These results demonstrate that expression of these PRRs in tomato and potato can generate ethylene specifically upon their cognate PAMPs treatment. Due to there being no time to finish the pathogen assay at the end of this thesis work progress, the resistance conferred by EFR, RLP23, and RLP42 in tomato and potato remains to be investigated.

Results

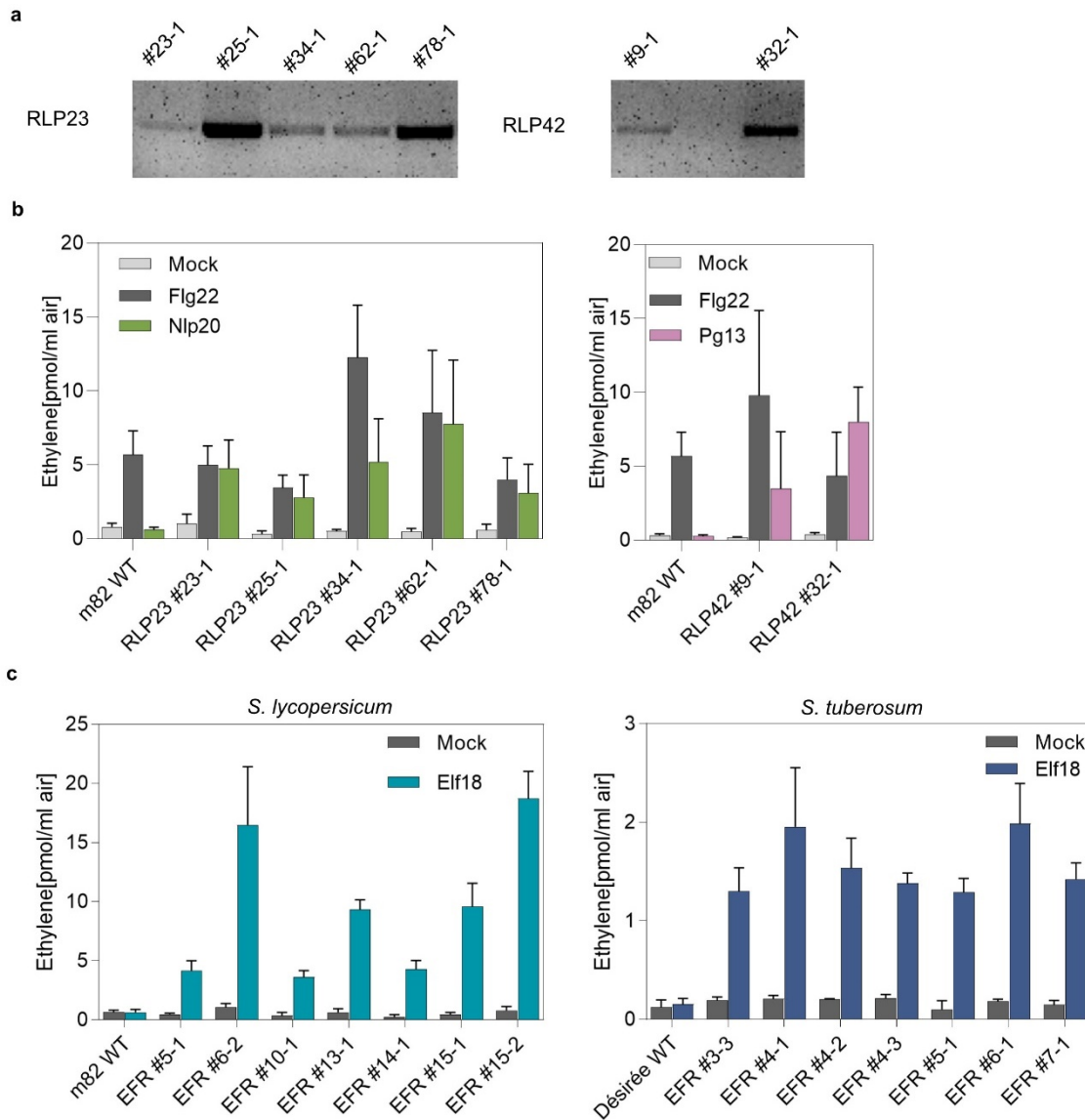


Figure 3.25. Identification of RLP23, RLP42, and EFR stable transformation in tomato and potato.

a, PCR amplification of *RLP23* and *RLP42* gene fragments using genomic DNA extracted from T1 generation leaves transformed with *RLP23* and *RLP42* by specific primers. **b**, Ethylene production in tomato leaf discs expressed with *RLP23* and *RLP42* receptors 4 h after treatment with water (mock), 5 μ M flg22 (positive control), 5 μ M nlp20 or 5 μ M pg13. Bars represent means + S.D. of three replicates. **c**, Leaf discs cut from wild type and EFR transgenic tomato and potato lines were incubated with water (mock), 1 μ M elf18. Bars represent means + S.D. of three replicates.

4 Discussion

The main goal of this thesis work was to investigate the feasibility of stable expression of stacked PRRs from crops into model plants, *A. thaliana* and *N. benthamiana*, to gain broad-spectrum and/or robust resistance. Transgenic expression of receptors CORE, ELR, EIX2, and CORE-ELR, CORE-ELR-EIX2 cassettes in *Arabidopsis* confers perception of corresponding ligand treatments. Defense responses such as ROS burst, ethylene production, and HR were activated and detected. Expression of these receptors contributed to enhanced resistance to pathogens related to corresponding recognition species. Furthermore, the CORE-ELR-EIX2 cassette expression confers broad-spectrum resistance to three pathogen species, which indicates that stacking PRRs in *Arabidopsis* is sufficient to elicit defense response by PAMPs treatment and broad-spectrum resistance. In *N. benthamiana*, the stable expression of receptor CORE, FLS3, EIX2, and CORE-FLS3, CORE-FLS3-EIX2 cassettes leads to sensitivity to corresponding PAMPs and immune responses as well. While expression of CORE-FLS3 cassette does not confer robust resistance to bacteria *Pstab*, although the ROS and ethylene productions elicited by csp22+flgII-28 are significantly higher than csp22/flgII-28 solely treated. Unexpectedly, CORE-FLS3-EIX2 expressing plants show statistically significant resistance to bacterial infection compared to either single CORE/FLS3 or simultaneous CORE-FLS3 expression. These results suggest that the combination of CORE and FLS3 confers similar resistance compared to individual CORE/FLS3 expressing lines, while EIX2, a fungal receptor, has a potential function in CORE-FLS3-EIX2 expressing lines showing robust resistance to bacteria.

Since the expression of PRRs confers resistance to pathogens in model plants, PRRs from *Arabidopsis* including EFR, RLP23, and RLP42 are chosen for stable transformation in tomato and potato. The ethylene production elicited by cognate PAMPs in transgenic lines is detected and disease resistance remains to be investigated.

To investigate the susceptibility of multiple PRRs mutants in *Arabidopsis*, the quintuple mutant *efr fls2 cerk1 lym3 sobir1* is generated by crossing and infected with *Pst* DC3000 and *B. cinerea*. The susceptibility of quintuple mutants to *Pst* DC3000 is statistically identical to double mutant *efr fls2*, while to *B. cinerea* is more susceptible compared to double mutant *efr fls2*, triple mutants *efr fls2 sobir1* and *efr fls2 cerk1*, demonstrating that these PRRs have different additive functions in resistance to bacteria and fungi.

4.1 Stacking PRRs in model plants is a powerful approach for gaining broad-spectrum resistance

Plant disease can cause loss and damage in crops during the whole growth and development phase leading to threatened yield and quality. According to a United Nations report, the global population reached 8 billion in 2022, while the crop losses brought by plant disease and pests are in the estimated range of 10%-40% in the world. The application of traditional chemical pesticides is effective in decreasing losses, however, it is followed by harmfulness to the environment and human health. The disease caused by pathogens can be controlled with engineering crop resistance, especially in this marvel era in which biotechnologies are emerging endlessly, and resistance genes are constantly discovered.

In the conventional strategy of resistance breeding, the gained resistance in cultivars is conferred by the introgression of resistant loci from wild species. To save breeding time and enhance the accuracy of interested gene expression, QTLs and immune receptors are generally cloned or modified in crops like wheat, maize, and potato. Plants employ a two-tiered immune system, PTI and ETI, to fend off pathogen infection. The receptors involved in these two layers, PRRs and NLRs, are assigned as assets of genetic engineering for inter-species transformation in plants to provide resistance.

4.1.1 The comparison of PRRs with NLRs in plant breeding

Several examples of ectopic expression of pattern recognition receptor genes leading to improved resistance have been reported so far. The notable employment of PRRs in genetic engineering is transgenic expression of Arabidopsis receptor EFR into crops which enhances resistance to phytopathogenic bacteria. Other PRRs like rice Xa21 and Arabidopsis RLP23 are also well-known for their ectopic expression in crops to reduce symptoms and colonization in pathogen infection (Albert et al., 2015; Holton et al., 2015; Schwessinger et al., 2015). Since the PAMPs are frequently conserved among pathogen races, the resistance conferred by PRRs is theoretically durable. These results indicate that PRRs that recognize PAMPs derived from a wide range of pathogens are instrumental in increasing resistance and engineering immunity in plants.

Although disease resistance (R) genes are widely applied for traditional breeding to confer resistance, NLRs encoded by R genes are challenging in interfamily transfer due to the interacting and signaling downstream components being different between distinct families. While there are successful cases as well, transfer of Arabidopsis RRS1/RPS4 is functional not only in other Brassicaceae plants, *Brassica rapa*, and *Brassica napus*, but also in Solanaceae plants, *Nicotiana benthamiana*, and *Solanum lycopersicum*, rendering resistance to pathogens (Narusaka et al., 2013). To avoid the autoimmune response during the expression of NLRs, pepper Bs2 is transferred into sweet orange driven by a pathogen-

inducible promoter to provide immunity (Sendín et al., 2017). As the recognition of specific effectors by NLRs is the key point in ETI, once the effector genes are mutated or lost, the resistance conferred by transgenic NLRs is eventually deprived. Hence, the selection of proper NLRs that can sense core or wide-spread effectors in pathogens is vital for breeding.

Considering these cases, defects of NLRs and abundant availability of PRRs, crop PRRs SICORE, SmELR, SIFLS3, and SIEIX2 were chosen for testing the hypothesis that ectopic expression of stacking PRRs is sufficient to gain resistance against pathogens in model plants.

4.1.2 Gene stacking in breeding requires less labor and time

Even though the interfamily transfer of a single PRR is effective in conferring a certain extent of resistance against one pathogen species, it is indispensable for breeding to gain broad-spectrum/robust resistance to pathogens in plants accounting for exposure to a variety of pathogens. To overcome time-consuming introgression for the combination of R genes or QTLs, utilization of pyramided resistance genes by genetic engineering in breeding is able to detect different pathogens or isolates of one pathogen population and able to combine several genes at the same time to prevent potential loss efficiency caused by pathogen mutations.

Integrating multiple R genes has not been exclusive for altering crop resistance. For example, a five-gene cassette consisting of five R genes on one vector transformed into wheat provided resistance to fungal pathogens (Luo et al., 2021). Recently, a study showed that stacking PERU and RLP23 in potato confers more resistance against notorious *Phytophthora infestans* (Ascurra et al., 2023). Therefore, stacking genes on one vector is an efficient and effective way to integrate multiple resistance genes and gain higher resistance simultaneously. During the cloning process, unlike the five-gene cassette mentioned above without any tag fusion, the PRRs chosen for this study are driven by different promoters and fused with different tags to prevent gene silencing and undetectable expression.

Another advantage of gene stacking on one vector for transformation is that the combination of different receptor genes is just like building blocks, every receptor with its promoter and tag is considered a block for preparation. Combinations can be assembled for any purpose like stacking receptors recognizing PAMPs that originated from different pathogen species, or receptors recognizing PAMPs derived from one pathogen species. The rapid and easy operation enables the stacking of PRRs to facilitate the improvement of disease resistance, however, in this study, the PRR gene cassettes contain a maximum of 3 receptors (CORE-ELR-EIX2 and CORE-FLS3-EIX2), whether pyramiding of more PRR genes will affect expression and immune output is unknown.

4.2 CORE-ELR-EIX2 gene cassette confers broad-spectrum resistance in Arabidopsis

The expression of CORE, ELR, and EIX2 which recognize bacterial, oomycete and fungal PAMPs respectively confer immune responses such as ROS and ethylene production in Arabidopsis. Expression of stacking gene cassettes, CORE-ELR and CORE-ELR-EIX2, provides broad-spectrum resistance to infection of *Pst* DC3000, *B. cinerea*, and *A. laibachii*. It elucidates that the expression of a single receptor and stacked receptors can confer defense response in Arabidopsis, and the employment of stacking PRRs is sufficient for engineering broad-spectrum resistance against pathogens.

4.2.1 PRRs do not function boundlessly in heterologous expression

Constitutively expressing CORE, ELR, EIX2, CORE-ELR, and CORE-ELR-EIX2 in Arabidopsis enables recognition of corresponding PAMPs and enhanced and broad-spectrum resistance in a ligand-independent way. The activation of defense responses such as ROS burst, ethylene accumulation, and HR in Arabidopsis transgenic plants by corresponding PAMPs proves that the immune signaling cascade required by CORE, ELR, and EIX2 is conserved in these plant species. Without priming by elicitors, transgenic plants exhibit resistance to *Pst* DC3000 infection which is an advantage in plant breeding.

Although the selected CORE, ELR, and EIX2 can function well in the ectopic expression of Arabidopsis, it does not represent that every PRR can achieve this. The alike crop receptors, SIFLS3 identified from *S. lycopersicum* binding flgII-28 and PERU from *S. tuberosum* sensing Pep-13, were not found as efficient as the receptors stacked in this research. Introduction of SIFLS3 and PERU respectively in Arabidopsis can not cause any immune response when treated with cognate ligands flgII-28 and Pep-13 (Supplementary Figure 7.5).

The observation of SIFLS3 transgenic lines in Arabidopsis without ROS burst induced by flgII-28 is consistent with previous research (Hind et al., 2016). The research shows that SIFLS3 can recruit AtBAK1 dependent on flgII-28 treatment and enhance ROS burst when co-expressed with AtBAK1 in VIGS-silenced *NbBAK1* plants. Hence, it's intriguing that SIFLS3 employs AtBAK1 in vitro, whereas the stable transformation of SIFLS3 has no immune outputs in Arabidopsis. There is another study that reports that the comparison of SIFLS3 kinase activities with SIFLS2 has shown that the TM and KD regions are important for kinase activity and ROS response (Roberts et al., 2020). Probably, SIFLS3 functions in another signal pathway different from that utilized by AtFLS2 in Arabidopsis, which may account for the evolution of SIFLS3 in Solanaceous families while it is absent in Brassicaceae.

PERU is an LRR-RK in potato that recognizes the Pep-13 ligand produced by oomycete species (Torres Ascurra et al., 2023). Upon Pep-13 treatment, PERU recruits SERK3A, an ortholog of AtBAK1, in *N. benthamiana* and conducts immune response. However, we observed that the stable transformation of PERU in Arabidopsis leads to no ethylene production even though PERU protein accumulation is detectable. Potentially these results indicate that in the distinct families, both SIFLS3 and PERU cannot interact with molecular components and/or downstream signaling which are conserved specifically. The co-receptors SOBIR1, RLK BAK1, and other RLCKs are crucial for mounting downstream immune response therefore cognate co-receptors or signaling components may be easily accessible for binding to PRRs intimately and transducing signals. The reason why SIFLS3 and PERU have no defense response in transgenic Arabidopsis remains to be elucidated.

4.2.2 ROS burst triggered by xylanase in Arabidopsis is EIX2-independent

Unlike the ROS burst triggered by xylanase in *N. benthamiana* is EIX2-dependent, xylanase can elicit ROS production in Col-0 plants with two phases which are always associated with ETI. However, ROS burst elicited by xylanase remains in *eds1*, *pad4*, *adr1 triple*, *nrg1 double*, and *helperless* mutants. Then the leucine-rich repeat family protein mutant library, *sobir1* mutant and *efr fls2 cerk1 lym3* quadruple mutants were treated with xylanase. The *rlp6* and *rlp13* mutants show no ROS production upon xylanase while *sobir1* mutant retains ROS burst.

In Arabidopsis, multiple LRR-RPs, but not all, like RLP23, RLP30, RLP32, and RLP42 function with the co-receptor SOBIR1 leading to no immune response in the *sobir1* mutant upon cognate PAMPs treatment (Albert et al., 2015; Fan et al., 2022; Zhang et al., 2014; Zhang et al., 2013). However, the ROS burst elicited by xylanase is abolished in *rlp6* and *rlp13* mutants but not in *sobir1* mutant indicating that SOBIR1 is not required for the ROS response to xylanase. It might be possible that some RLPs in Arabidopsis function in a SOBIR1-independent manner, or, alternatively that RLP6 or RLP13 are not genuine xylanase receptors and only associated with the recognition of xylanase.

The protein xylanase from *Trichoderma viride* is ordered from company Sigma-Aldrich and is contaminated with cellulase, β -glucosidase, and β -xylosidase with contaminant activities of < 1%, $\leq 0.1\%$, and $\leq 0.1\%$, respectively. To identify the immunogenic pattern in this mixture, HPLC purification should be employed to distinguish which components display immunogenic activity.

4.2.3 Broad-spectrum resistance conferred by perception of conserved PAMPs

PAMPs play key roles in microorganism subsistence and plant innate immunity. Cold shock protein, purified from *M. lysodeikticus*, comprises epitope csp22 which is sensed by CORE in tomato and present widely in bacteria including *Pseudomonas* species for adapting to cold

stress (Craig et al., 2021; Felix & Boller, 2003). In oomycetes, elicitin is a structurally conserved protein in *Phytophthora*, *Pythium*, *Pseudoperonospora*, *Hyaloperonospora*, and *Albugo* species which is recognized by ELR in potato leading to cell death (Kharel et al., 2021; Links et al., 2011). Endoxylanase, termed as ethylene-induced xylanase (EIX) and belonging to glycosyl hydrolase family 11, exists in many fungi including *Botrytis*, is isolated from fungus *Trichoderma viride* and perceived by EIX1 and EIX2 in tomato (Frías et al., 2019). As conserved structures or components of pathogen cell walls or survival strategies, PAMPs are settled to exist in pathogens with little possibility of mutation which facilitates identification by PRRs. To take advantage of this character, interfamily transfer of PRRs can enhance potential resistance to one pathogen species or even several species. Stable transformation of RLP23 in potato confers broad resistance to fungi and oomycetes due to the microbial protein NLP found in these pathogen species (Albert et al., 2015; Böhm et al., 2014). Moreover, stacking PRRs that recognize distinct pathogen species in crops is the speedy way to simultaneously possess broad-spectrum resistance against plenty of infection. In this work, pyramiding CORE-ELR-EIX2 in Arabidopsis can confer resistance against *Pst* DC3000, *A. laibachii*, and *B. cinerea* infection which demonstrates that the strategy of stacking PRRs is feasible for fending off a broad range of pathogen infections.

4.3 CORE-FLS3-EIX2 confer enhanced resistance to *Pstab* in *N. benthamiana* compared to CORE-FLS3

In this thesis work, we observed that bacterial-PAMP-recognising receptors CORE and FLS3 expressing plants conferred resistance to *Pstab* in *N. benthamiana*. However, the expression of CORE-FLS3 in *N. benthamiana* conferred similar resistance to *Pstab* infection as a single CORE- or FLS3-expressing plants. These two bacterial-PAMP-recognising receptors stacked with one fungal-PAMP-recognising receptor, CORE-FLS3-EIX2, enhanced resistance in transgenic plants compared with CORE-, FLS3-, and CORE-FLS3-expressing lines about a 10-fold difference in bacterial populations.

4.3.1 Additional immune outputs do not in parallel with robust resistance

In CORE-FLS2 expressing *N. benthamiana* line # 9, ROS burst and ethylene production elicited by csp22+flgII-28 were much higher than after either single ligand treatment. This phenomenon is observed as well in another recent study that PERU-RLP23 transgenic potato when treated with Pep-13 and nlp20 together showed an additive response in ROS and ethylene production (Ascurra et al., 2023). However, the simultaneous application of bacterial ligands, IF1 and elf18, caused neither additive nor synergistic ethylene response compared with individual treatment of IF1 or elf18 in Arabidopsis (Fan et al., 2022). These results suggest that some PAMPs can produce additive effects whereas certain PAMPs cannot in synchronized treatments.

In PTI, PRRs contribute to the recognition of PAMPs and participate in the formation of receptor complexes with co-receptors SOBIR1 and BAK1, activating downstream signaling by RLCKs and integrating multiple networks for immune outputs. The multi-layered immune system is subject to regulation by BRI1, U-box E3 ligases, and other regulators to maintain the balance between resting and defense status. ROS burst, as signaling molecules, activation of NADPH oxidase RBOHD is required for ROS production and subject to Ca²⁺-dependent protein kinases, RLCKs like PBL13 and BIK1 (Zhou & Zhang, 2020). However, the entire pathway controlling ROS production is currently unknown. Thus, the simultaneous processing of both ligands resulting in additive effects is still waiting to be revealed. Further experiments studying signaling pathways in PTI will help uncover the relationship between ligand treatment and immune outcomes.

Although the additive effects conferred by *csp22+flgII-28* treatment in the CORE-FLS3 expressing line # 9 is observed in this work, the resistance to bacterial infection is identical between stacking two bacterial receptors and one bacterial receptor. This suggests that the final level of disease resistance is not correlated to immune responses, at least in *Pstab* infection. In this result, the hypothesis that stacking of two bacterial PRRs CORE and FLS3 confers more resistance than one receptor expression is not experimentally achieved. However, in another research, stacking PERU and RLP23 was demonstrated that double receptors transformants show more resistance to *P. infestans* infection (Ascurra et al., 2023). To achieve the synergistic effects in disease resistance by stacking PRRs, selection of PRRs and further studies to uncover how PRRs act in stacking are crucial for crop breeding.

4.3.2 Potentiated effect of EIX2 in resistance to *Pstab* through CORE-FLS3-EIX2

Although expression of CORE-FLS3 confers no statistically significant difference in the growth of bacteria compared to plants expressing either CORE or FLS3 in *Pstab* infection, surprisingly, the involvement of another fungal receptor EIX2 leads to CORE-FLS3-EIX2 transgenic lines exhibiting significant resistance to bacterial infection compared to either single CORE/FLS3 or CORE-FLS3 expression in *N. benthamiana*, while expression of individual EIX2 confers no resistance to *Pstab* infection. These results indicate that EIX2 has a potential effect on enhancing bacterial resistance when co-expressed with CORE and FLS3.

In plant immunity, PTI and ETI potentiate each other mutually to produce a more robust immune response and resistance against pathogen infections is supported by several research reports (Chang et al., 2022). It is possible that in PTI, as recognition receptors of distinct pathogen species, they share same pathways and interact with each other to generate increasing resistance. A research reports that activation of FLS2 by *flg22* priming can induce juxtamembrane phosphorylation of CERK1 in the presence of BAK1, which

increases fungal resistance (Gong et al., 2019). This result reveals the crosstalk between sensing of bacterial PAMPs by PRRs and fungal resistance associated with CERK1. Likewise, flg22 perception also functions in antiviral signaling by inducing phosphorylation of NIK1 which regulates antiviral immunity as an RLK (Li et al., 2019). These reports demonstrate that the interactions of PRRs or PRRs with other RLKs potentiate plant defense against more classes of pathogen.

Plants deploy cell-surface and intracellular immune receptors to detect pathogen-derived PAMPs or effectors and activate plant immunity. It is intricate that the deployment of recognition receptors with signaling pathways and networks is integrated to generate immune outcomes rather than isolate activation. Incremental evidence reveals that PTI and ETI are required for each other and the crosstalk between PTI and ETI, PRRs and PRRs contributes to enhancing plant immunity. Stacking PRRs may not result in additive immunity because one receptor might be already sufficient to potentiate ETI. Plant innate immunity as an integration surveillance system employs all components to produce response and the important mechanism of potentiation remains to uncover in future work.

4.4 Loss of the majority of identified PRRs/co-receptors in Arabidopsis results in susceptibility to pathogen infection

To mutate most of the identified PRRs/co-receptors involved in PAMPs perception, quadruple mutants *efr fls2 cerk1 lym3* and triple mutants *efr fls2 sobir1* are crossed and quintuple mutants *efr fls2 cerk1 lym3 sobir1* are characterized. Ideally, if more receptors are mutated, Arabidopsis would be more susceptible to disease invasion. However, the pathogenicity assays show that in *Pst* DC3000 infection, quintuple mutants are identically vulnerable to double mutants *efr fls2*, whereas *B. cinerea* inoculation quintuple mutants are more susceptible than double and triple mutants, *efr fls2* and *efr fls2 sobir1*.

4.4.1 Quintuple mutants *efr fls2 cerk1 lym3 sobir1* are as susceptible as double mutants *efr fls2* against *Pst* DC3000 infection

In Arabidopsis, PTI employs LRR-RKs FLS2, EFR and XPS1, LRR-RPs RLP1 and RLP32, LecRK LORE, and LysM-RK LYM1/3 to perceive a range of PAMPs from bacteria involving bacteria secreted peptides, lipids, peptidoglycans, and polysaccharides (W. L. Wan et al., 2019). However, these identified pairs constitute a rather restricted list of PRRs/PAMPs based on the total number of LRR-RKs and LRR-RPs existing in Arabidopsis species. There are 226 LRR-RKs and 59 LRR-RPs in *A. thaliana*, so the discovered receptors are a drop in the bucket (Ngou et al., 2022). Quintuple mutants *efr fls2 cerk1 lym3 sobir1* do not exhibit anticipated overwhelming susceptibility to *Pst* DC3000 infection, which may be due to the abundance of bacterial receptors undiscovered in Arabidopsis.

The comparable susceptibility between quintuple mutants and double mutants (*efr fls2*) reveals that FLS2 as a bacterial receptor plays a dominant role in plant immunity. Flagellin widely exists in bacteria and contributes to bacterial movement in the flagellum for survival. In mammals flagellin is recognized by the Toll-like receptor TLR5, and in plants flg22 and flgII-28 derived from flagellin are sensed by FLS2 and SIFLS3, respectively (Chinchilla et al., 2006; Hayashi et al., 2001; Hind et al., 2016). The widespread flagellin receptors in plants indicate that the vital role of PAMP flagellin and FLS2 is an incomparable bacterial receptor. There is no other receptor like FLS2 that is widely distributed and elicits a robust immune response. It is therefore reasonable that FLS2 is a dominant bacterial receptor and exerts crucial effects on stomata during *Pseudomonas* infection compared to other bacterial receptors (Zeng & He, 2010). However, in this thesis work, the bacterial infection is tested with *Pst* DC3000 only, it is not known whether FLS2 acts as a dominant receptor for other phyto-bacteria or whether other bacterial receptors function intensely in other bacterial infections.

As a plant surveillance system, PTI utilizes cell surface PRRs to monitor the invasion of different pathogens. PRRs on the cell surface execute alarms, so the primary function is to warn the plant of approaching danger. This may be the reason why pyramiding CORE-FLS3 in *N. benthamiana* leads to similar resistance as single receptor CORE- or FLS3- expressing plants. Alarmed against a pathogenic species, once launched, the plants transduce signals and take immune actions. As for the number of alarms that sound, there may not be a significant difference.

4.4.2 Lack of CERK1 in Arabidopsis results in susceptibility to *B. cinerea* infection

Different from the LRR-RK type receptor FLS2, LysM type receptor CERK1 was associated with recognition of chitin in Arabidopsis and found in many other plants like rice, maize as well as Solanaceous plants (Yang et al., 2022). Similar to flagellin, chitin is an indispensable and highly conserved component in fungal cell walls which is released from fungal cell walls during fungal infection (Lenardon et al., 2010). In this thesis work, mutations of CERK1 lead to a significant susceptibility to *B. cinerea* infection which is vividly reflected in the diameter of the lesion. If FLS2 is regarded as a dominant bacterial receptor during *Pst* DC3000 infection, CERK1 is referred to as an incredibly crucial receptor/co-receptor during *B. cinerea* infection.

Based on the absence of FLS2 or CERK1 in mutant plant defense, we can conclude that the fundamental character of PAMPs in pathogen survival or in the infection cycle process determines the wide distribution and highly effective resistance of corresponding receptors in plants. Over millions of years, as plants moved from aquatic environments to lands, terrestrial plants were exposed through the interaction with microbes, leading to co-evolution between the plant and pathogen. Plants are constantly attacked by microbes, so the plant

immune system has evolved two layers of strategies to fend off invasion. On the extracellular aspect, recognition of PAMPs via PRRs in PTI signals to the host cell. This recognition results in the co-evolution of PRRs and PAMPs in which the PRRs sense essential and highly conserved components that are widespread in microbial species, while PRRs that perceive PAMPs that occur rarely or in pathogens from isolated regions tend to exist in some plant species only or are eventually lost. This co-evolution can enhance the function of dominant receptors in plant defense and select functional diversification of PRRs in plant species. This is perhaps an explanation for the different responsiveness to IF1 or PG in *Arabidopsis* or *Brassica* accessions (Fan et al., 2022; Zhang et al., 2021). The deployment of dominant receptors like FLS2, CERK1, and other receptors in plants facilitates the recognition of main PAMPs which can be used to engineer robust and broad-spectrum resistance against disease.

4.5 The developmental tendency of PRR stacking in resistance breeding

In this thesis work, the stacking CORE-ELR-EIX2 confers broad-spectrum resistance to infection in *Arabidopsis* and the expression of CORE-FLS3-EIX2 in *N. benthamiana* leads to enhanced resistance to bacterial infection, demonstrating that pyramiding PRRs in breeding is indispensable and a possibility in breeding for enhanced resistance. However, there are still some approaches to improve this strategy. To overcome possible incompatibilities during interfamily transfer, application of chimeric receptors with swapped kinase domains can transduce signaling and more efficiently elicit immunity. Examples for this are chimeric receptors like EFR and XA21, ReMAX and EIX2 that confer recognition of elf18 or eMAX in *Arabidopsis* or *N. benthamiana* (Holton et al., 2015; Jehle et al., 2013).

According to this work, EIX2 confers limited immunity to *Arabidopsis* and *N. benthamiana*, at an early stage only. However, in nature the harassment of pathogen and infection process lasts constantly. One of the major goals in plant breeding is to create cultivars with durable resistance to more pathogens. Since NLRs in ETI possess reinforced resistance, the combination of PRRs and NLRs constitutes a mechanism for broad-spectrum resistance to multiple pathogen species and durable resistance to important effectors. Due to host-pathogen co-evolution, the studies on PRRs and NLRs not only provide insights into how plants can perceive microbe components or secreted effectors, but also present such an abundant source of PRRs and NLRs in different plant species that can be combined to improve and strengthen disease resistance.

Transgenic expression of PRRs in model plants confers resistance under laboratory conditions. However, the ultimate goal is to test the sustainability of transgenic plants in the field under natural and complex conditions. Transgenic expression of EFR in tomato was evaluated for bacterial disease resistance and total field yield (Kunwar et al., 2018). The

Discussion

phyllosphere of plants, including leaves, flowers, stems, fruits, and pollens, is colonized by numerous microbes containing commensals and pathogens. The phyllosphere microbiota homeostasis depends on host-microbe, microbe-microbe, and environmental interactions, furthermore, the disruption of this community by mutants or transgenic plants may lead to altered microbiota levels and even affect plant and human health. Higher-order mutants in receptors, particularly the *rbohD* mutant, caused alteration of phyllosphere microbiota in *Arabidopsis* (Pfeilmeier et al., 2021), and transgenic expression of Bs2 in tomato impacted on phyllosphere communities, whereas EFR did not (Bigott et al., 2023). However, how the phyllosphere microbiota is influenced by mutants and transgenic plants overexpressing one or more PRRs needs to be further studied.

5 Summary

Interfamily transfer of PRRs into plants has been proven to gain enhanced resistance to pathogen infections. Therefore, PRR stacking is considered a promising strategy for engineering immunity in crops. In this study, PRRs from crops, SICORE, SmELR, SIEIX2, and SIFLS3, were chosen for stacking and transferred into model plants *A. thaliana* and *N. benthamiana*, EFR, RLP23, and RLP42 were transferred into tomato and potato, conferring resistance to pathogens. Higher-order mutants lacking multiple PRRs in Arabidopsis were generated and were susceptible to pathogen infection.

In this study, constructs of SICORE, SmELR, SIEIX2 as well as stacking receptors SICORE-SmELR, SICORE-SmELR-SIEIX2 were generated and transferred into *A. thaliana*. SICORE-, SmELR-, and SIEIX2-expressing Arabidopsis exhibited ROS burst, ethylene production, and HR to cognate PAMP activation and enhanced resistance to pathogens *Pst* DC3000, *A. laibachii*, and *B. cinerea*, respectively. Stacking of SICORE-SmELR and SICORE-SmELR-SIEIX2 in Arabidopsis showed similar immune responses to plants expressing a single receptor and broad-spectrum resistance to these three pathogen species. Constructs of SICORE, SIFLS3, SIEIX2 as well as pyramid receptors SICORE-SIFLS3, SICORE-SIFLS3-SIEIX2 were generated and transferred into *N. benthamiana*. Expression of SICORE, SIFLS3, and SIEIX2 in *N. benthamiana* exhibited ROS burst, ethylene accumulation, and HR to cognate PAMP elicitation and increased resistance to pathogens *Pstab* and *B. cinerea* but not to *P. capsici*. SICORE-SIFLS3-expressing *N. benthamiana* showed identical resistance as SICORE/SIFLS3-expressing plants to *Pstab* infection instead of additional resistance, however, expression of SICORE-SIFLS3-SIEIX2 contributed more resistance to *Pstab* than plants expressing SICORE/SIFLS3 or SICORE-SIFLS3. Stable transformation of EFR, RLP23, and RLP42 conferred ethylene production in tomato and potato upon cognate PAMP treatments, and resistance to pathogens remained to be tested.

The quintuple mutant *efr fls2 cerk1 lym3 sobir1* was generated by crossing and exhibited similar susceptibility in *Pst* DC3000 infection compared to double mutant *efr fls2* and triple mutant *efr fls2 sobir1*, while was significantly colonized by *B. cinerea* in comparison with double and triple mutants.

These findings of broad-spectrum resistance conferred by PRRs stacking can support that pyramiding PRRs in plants is an effective approach to achieving broad-spectrum resistance in disease control and crop breeding.

6 Zusammenfassung

Es wurde nachgewiesen, dass der familienübergreifende Transfer von PRRs in Pflanzen zu einer erhöhten Resistenz gegen Pathogeninfektionen führt. Daher wird die Stapelung von PRRs als vielversprechende Strategie zur Entwicklung der Immunität in Nutzpflanzen angesehen. In dieser Studie wurden PRRs aus Nutzpflanzen, SICORE, SmELR, SIEIX2 und SIFLS3, für die Pyramide ausgewählt und in die Modellpflanzen *A. thaliana* und *N. benthamiana* übertragen, EFR, RLP23 und RLP42 wurden in Tomaten und Kartoffeln übertragen, was ihnen Resistenz verleiht gegen Krankheitserreger. Es wurden Mutanten höherer Ordnung erzeugt, denen mehrere PRRs in Arabidopsis fehlen und die anfällig für eine Pathogeninfektion sind.

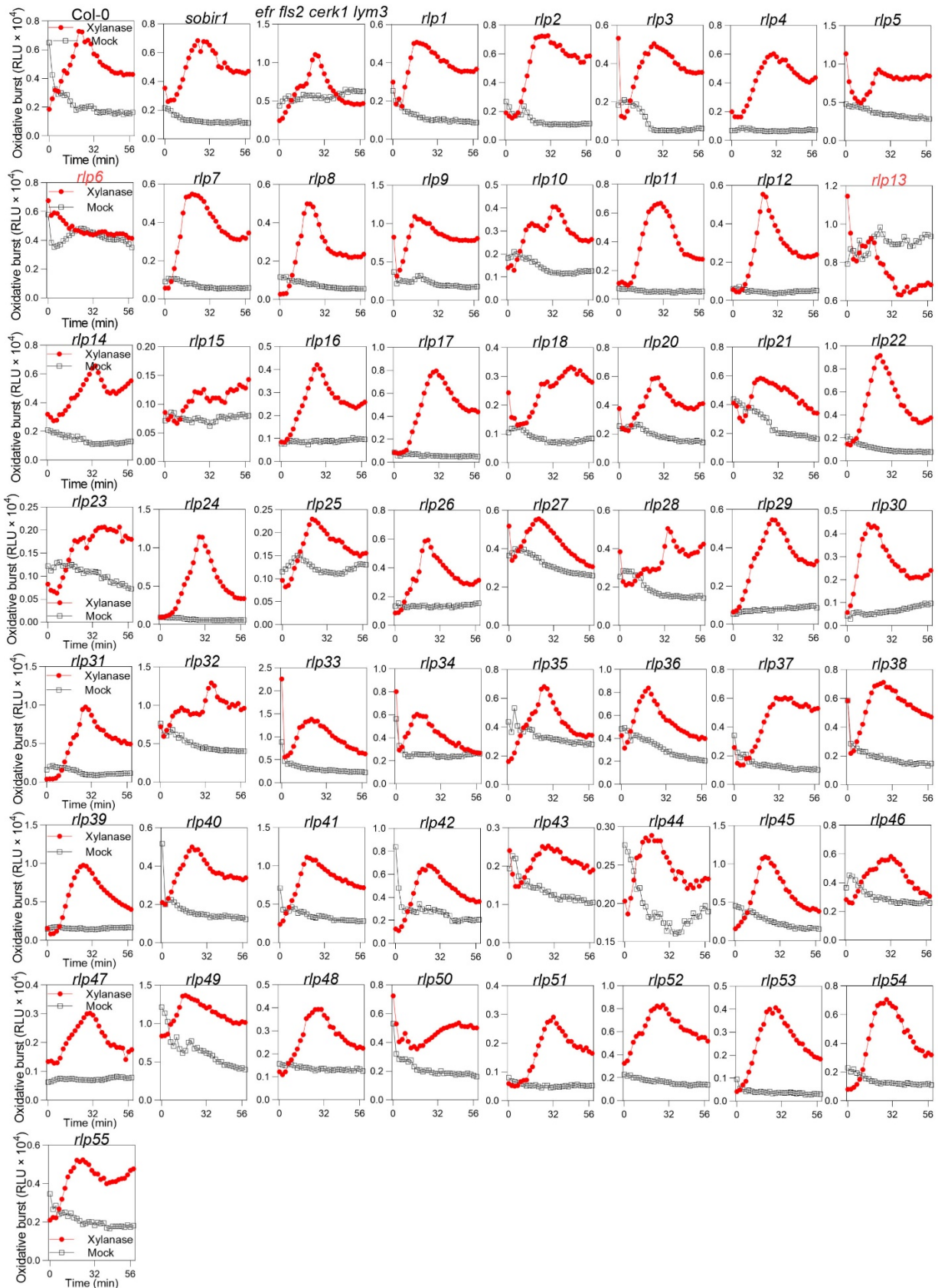
In dieser Studie wurden Konstrukte von SICORE, SmELR, SIEIX2 sowie den Stapelrezeptoren SICORE-SmELR, SICORE-SmELR-SIEIX2 generiert und in *A. thaliana* übertragen. SICORE-, SmELR- und SIEIX2-exprimierende Arabidopsis zeigten einen ROS-Burst, eine Ethylenproduktion und eine HR, die mit der PAMP-Aktivierung und einer erhöhten Resistenz gegen die Krankheitserreger *Pst* DC3000, *A. laibachii* bzw. *B. cinerea* einhergehen. Die Stapelung von SICORE-SmELR und SICORE-SmELR-SIEIX2 in Arabidopsis zeigte ähnliche Immunreaktionen auf Pflanzen, die einen einzelnen Rezeptor exprimierten, und eine Breitbandresistenz gegen diese drei Krankheitserregerarten. Konstrukte von SICORE, SIFLS3, SIEIX2 sowie der Pyramidenrezeptoren SICORE-SIFLS3, SICORE-SIFLS3-SIEIX2 wurden generiert und in *N. benthamiana* übertragen. Die Expression von SICORE, SIFLS3 und SIEIX2 in *N. benthamiana* zeigte einen ROS-Burst, eine Ethylenakkumulation und HR, die mit der PAMP-Auslösung und einer erhöhten Resistenz gegen die Krankheitserreger *Pstab* und *B. cinerea*, jedoch nicht gegen *P. capsici*, einhergehen. SICORE-SIFLS3-exprimierende *N. benthamiana* zeigten eine identische Resistenz gegenüber *Pstab*-Infektionen wie SICORE/SIFLS3-exprimierende Pflanzen anstelle einer zusätzlichen Resistenz. Allerdings trug die Expression von SICORE-SIFLS3-SIEIX2 zu einer stärkeren Resistenz gegen *Pstab* bei als Pflanzen, die SICORE/SIFLS3 oder SICORE-SIFLS3 exprimierten. Eine stabile Transformation von EFR, RLP23 und RLP42 führte bei verwandten PAMP-Behandlungen zu einer Ethylenproduktion in Tomaten und Kartoffeln, und die Resistenz gegen Krankheitserreger musste noch getestet werden.

Die Fünffachmutante *efr fls2 cerk1 lym3 sobir1* wurde durch Kreuzung erzeugt und zeigte eine ähnliche Anfälligkeit bei *Pst* DC3000-Infektionen im Vergleich zum Doppelmutanten *efr fls2* und dem Dreifachmutanten *efr fls2 sobir1*, während er im Vergleich zu Doppel- und Dreifachmutanten signifikant von *B. cinerea* besiedelt wurde.

Zusammenfassung

Diese Ergebnisse der durch die Stapelung von PRRs hervorgerufenen Breitbandresistenz können belegen, dass die Pyramidenbildung von PRRs in Pflanzen ein wirksamer Ansatz zur Erzielung einer Breitbandresistenz bei der Krankheitsbekämpfung und Pflanzenzüchtung ist.

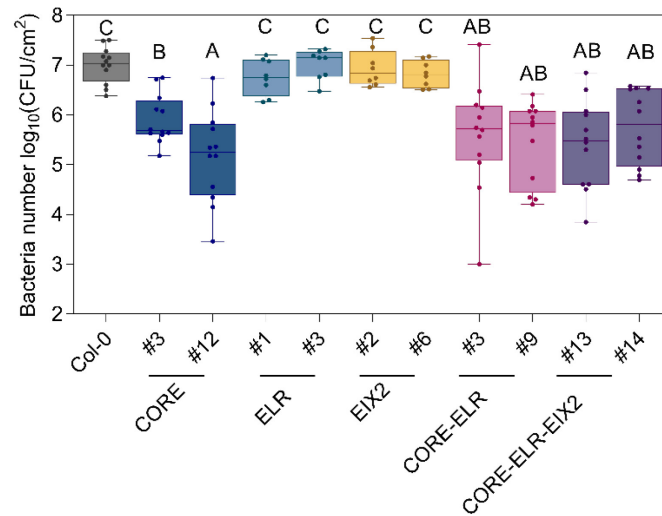
7 Supplementary



Supplementary Figure 7.1. ROS burst caused by xylanase in Arabidopsis mutants.

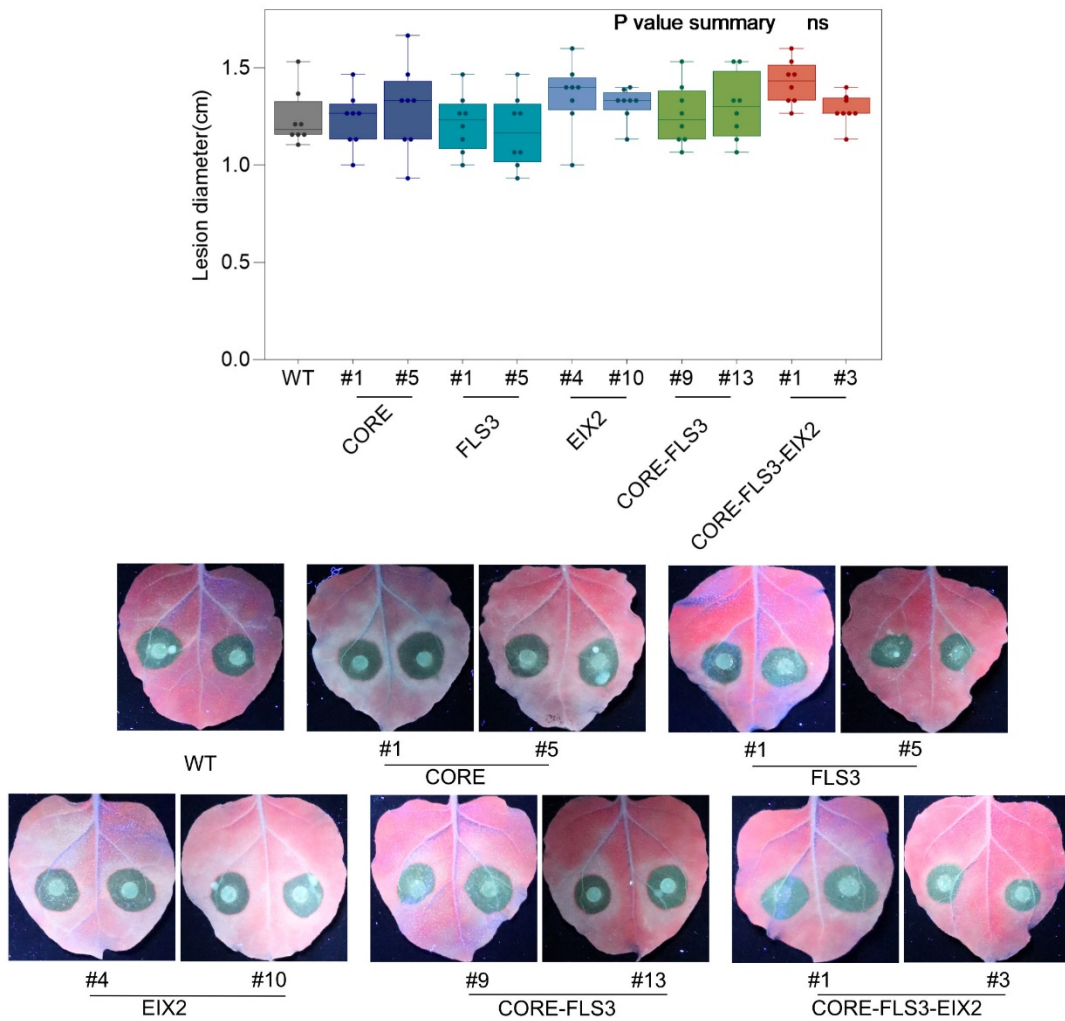
Leaf discs from Col-0, 54 *rlp* mutants, *sobir1*, and *efr fls2 cerk1 lym3* mutants were treated with MilliQ water (mock, as a negative control) and 5 μ M xylanase. ROS productions are visualized by luminol

kinetic curves. The mutants *rlp6* and *rlp13* (in red font color) have no ROS response to xylanase treatment.



Supplementary Figure 7.2. CORE-expressing Arabidopsis plants enhance resistance to *Pst* DC3000.

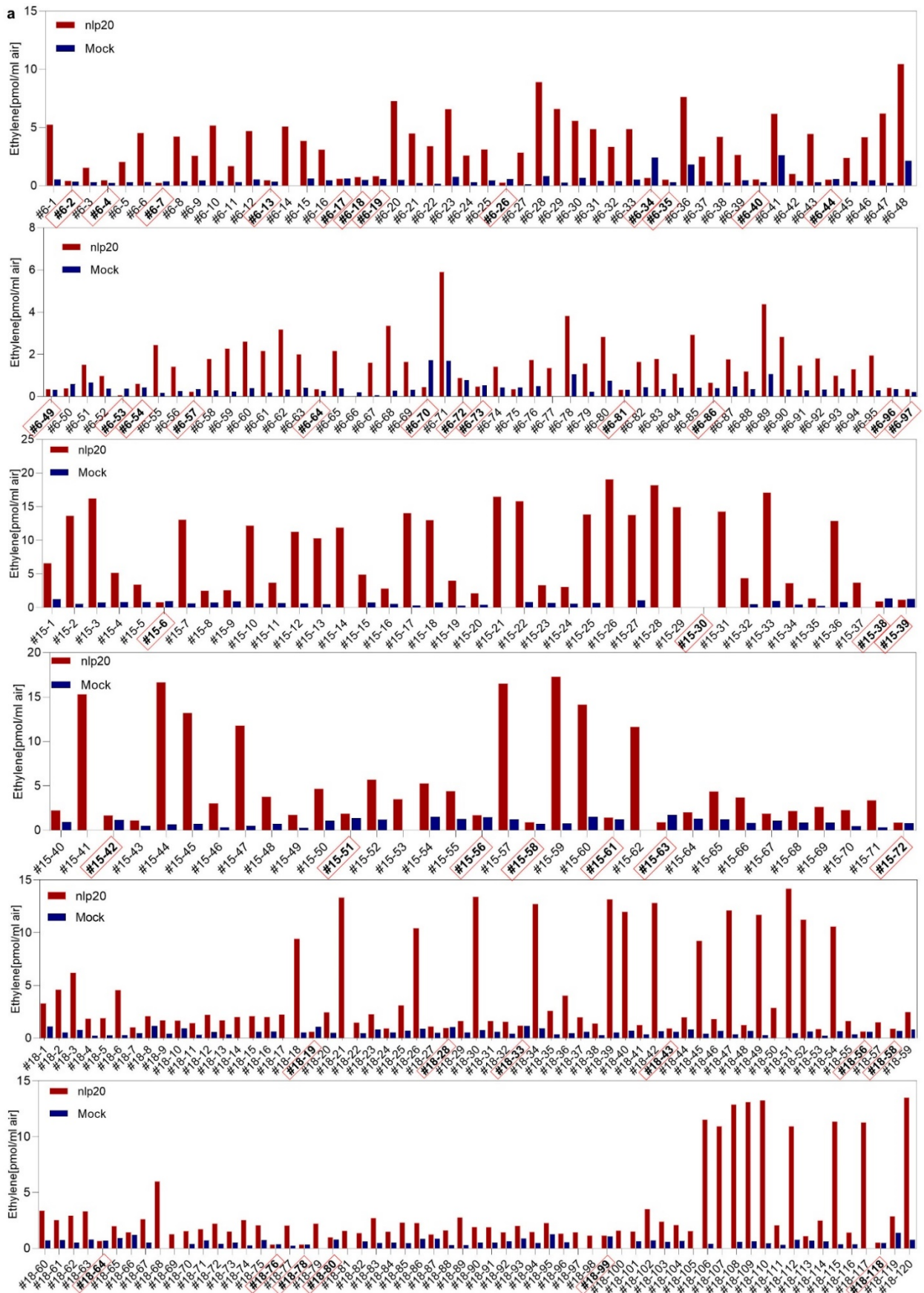
Col-0 and transgenic lines expressing CORE, ELR, EIX2, CORE-ELR, and CORE-ELR-EIX2 were sprayed with 1×10^7 cfu/ml *Pst* DC3000. Bacteria were quantified in extracts of leaves at 3 d post-infiltration. Data points are indicated as dots from three independent experiments (n=8). Box plots show the minimum, first quartile, median, third quartile, and maximum of \log_{10} cfu/cm² leaf tissues. Different letters above the box blot at 3 dpi indicate statistically significant differences among homogenous groups following Duncan's one-way ANOVA ($P < 0.05$).



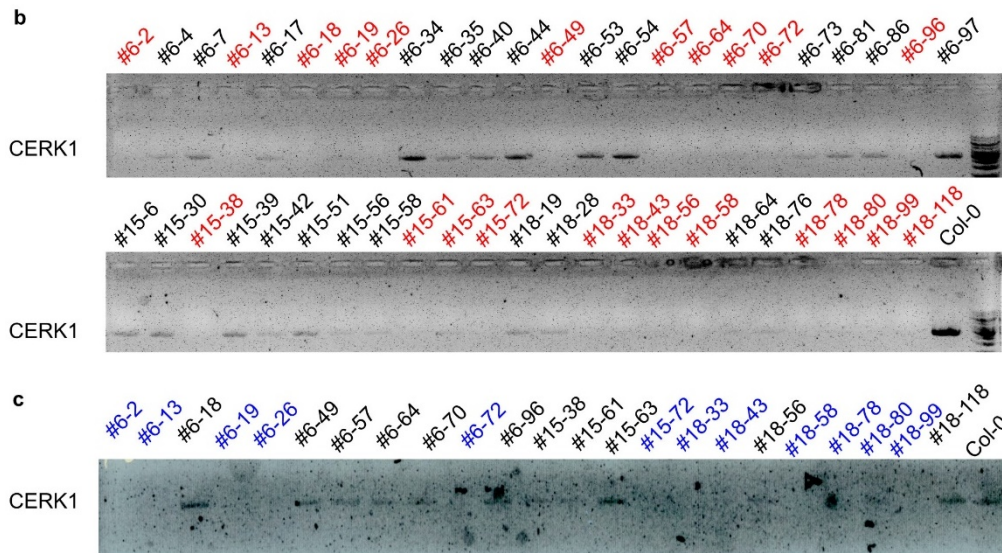
Supplementary Figure 7.3. CORE-, FLS3-, and EIX2-expressing *N. benthamiana* plants have no resistance to *P. infestans*.

The transgenic and wild-type plants were inoculated with 0.5 cm² *P. infestans* hyphae plugs on the two sides of the midrib. Lesion development was evaluated under UV light, and lesion diameter was measured by ImageJ at 2 dpi. Data points are indicated as dots from three independent experiments (n=8). Box plots show the minimum, first quartile, median, third quartile, and a maximum lesion diameter. Letter ns above the box blot indicates statistically non-significant differences among homogenous groups following Duncan's one-way ANOVA (ns $P > 0.05$).

Supplementary

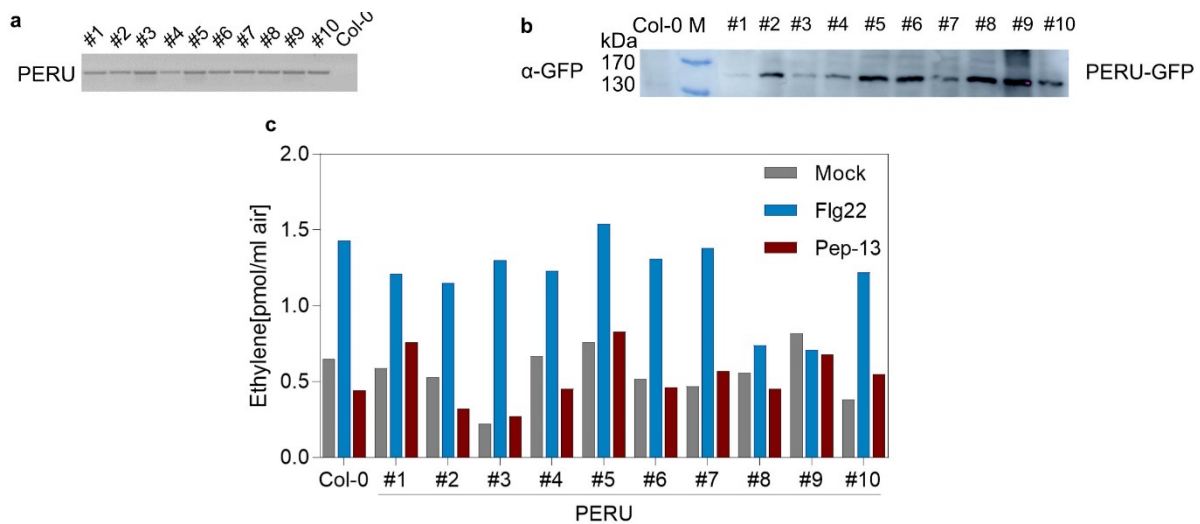


Supplementary



Supplementary Figure 7.4. Screening analysis of F2 progeny in the ethylene production assay and genotyping.

a, Leaf discs cut from F2 progeny plants were incubated with water (mock), 1 μ M nlp20. The 47 lines marked by red rectangles and bold texts have no ethylene production to nlp20 treatment and were utilized for subsequent genotyping screening. **b,c**, PCR amplification of CERK1 gene fragments using genomic DNA extracted from F2 lines leaves with CERK1 gene primers. **(b)** Firstly the 47 lines were screened by one pair of CERK1 gene primers and 23 lines in red color without bands. **(c)** These 23 lines were conducted by PCR amplification with another pair of CERK1 gene primers and 12 lines in blue color without bands were executed with next screening. The genomic DNA of Col-0 served as a negative control.



Supplementary Figure 7.5. Characterization of PERU receptor stable transgenic lines in *A. thaliana*.

a, PCR amplification of *PERU* gene fragments using genomic DNA extracted from T1 generation leaves transformed with *PERU*. The genomic DNA of Col-0 served as a negative control. **b**, The protein accumulation of *PERU*-GFP in T1 generation leaves was determined by Western blot with the α -GFP antibody. Total protein from Col-0 (left side of marker, M) served as negative controls. **c**, Leaf discs from Col-0 and transgenic lines were treated with MilliQ water (mock, as a negative control), 1 μ M flg22 (as a positive control), 100 nM Pep-13 and incubated on a shaker for 4 h before measurement.

8 Abbreviations

ADR1	Activated Disease Resistance 1	MS	Murashige and Skoog
BAK1	BRI1-associated Kinase1	NLR	Nucleotide-binding Leucine-rich Repeat
BIK1	<i>Botrytis</i> -induced Kinase 1	nM	Nanomolar
BIR1	BAK1-interacting Receptor-like Kinase 1	NRC	NLR Required for Cell Death
BKK1	BAK1-like Kinase 1	NRG1	N Requirement Gene 1
BRI1	Brassinosteroid Insensitive 1	PAD4	Phytoalexin Deficient 4
BSK1	BR-Signaling Kinase 1	PAMPs	Pathogen Associated Molecular Patterns
CERK1	Chitin Elicitor Receptor Kinase 1	PCR	Polymerase Chain Reaction
CNL	Coiled-coil NLR	PDA	Potato Dextrose Agar
CORE	Cold Shock Protein Receptor	PERU	Pep-13 Receptor Unit
Csp	Cold Shock Protein	PGN	Peptidoglycan
DAMPs	Damage Associated Molecular Patterns	PRRs	Pattern Recognition Receptors
DMSO	Dimethylsulfoxide	PTI	Pattern- triggered Immunity
DNA	Deoxyribonucleic Acid	QTL	Quantitative Trait Loci
DTI	Damage-associated Molecular Pattern-triggered Immunity	RBOHD	Respiratory Burst Oxidase Homolog Protein D
EDS1	Enhanced Disease Susceptibility 1	RLCKs	Receptor Like Cytoplasmic Kinases
EFR	Elongation Factor EF-Tu Receptor	RLK	Receptor Like Kinase
EIX	Ethylene-inducing Xylanase	RLP	Receptor Like Protein
EIX1/2	Ethylene-inducing Xylanase Receptor1/2	RLP1/ReMAX	Receptor of eMAX
ELR	Elicitin Response Protein	RNA	Ribonucleic Acid
ETI	Effector-triggered Immunity	RNL	RPW8-NBS-LRR
ETS	Effector-triggered Susceptibility	ROS	Reactive Oxygen Species
FLS2	Flagellin Sensing 2	Rpm	Revolutions Per Minute
FLS3	Flagellin Sensing 3	RT	Room Temperature
HR	Hypersensitive Response	RXEG1	Response to XEG1
INF1	<i>Phytophthora infestans</i> Elicitin 1	SA	Salicylic Acid
LORE	Lipooligosaccharide-specific Reduced Elicitation	SAG101	Senescence Associated Gene 101
LRR	Leucine-rich Repeat	SCP ^{Ss}	Small Cysteine-rich Protein <i>Sclerotinia sclerotiorum</i>
LYK4/5	LysM Containing Receptor-like Kinase	SERK	Somatic Embryogenesis Receptor-like Kinase
LysM	Lysin Motif	SOBIR1	Suppressor of BIR1
LYM1/3	LysM Protein 1/3	TBS	Tris-buffered Saline
MAPK	Mitogen Activated Protein Kinase	TNL	TIR-type Sensor NLR
mM	Millimolar	WT	Wildtype

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