

The Receptor-Like Protein ReMAX of *Arabidopsis* Detects the Microbe-Associated Molecular Pattern eMax from *Xanthomonas*^W

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As part of their immune system, plants have pattern recognition receptors (PRRs) that can detect a broad range of microbe-associated molecular patterns (MAMPs). Here, we identified a PRR of *Arabidopsis thaliana* with specificity for the bacterial MAMP eMax from xanthomonads. Response to eMax seems to be restricted to the *Brassicaceae* family and also varied among different accessions of *Arabidopsis*. In crosses between sensitive accessions and the insensitive accession Shakh dara, eMax perception mapped to *RECEPTOR-LIKE PROTEIN1* (*RLP1*). Functional complementation of *rlp1* mutants required gene constructs that code for a longer version of *RLP1* that we termed ReMAX (for receptor of eMax). ReMAX/*RLP1* is a typical RLP with structural similarity to the tomato (*Solanum lycopersicum*) RLP Eix2, which detects fungal xylanase as a MAMP. Attempts to demonstrate receptor function by interfamily transfer of ReMAX to *Nicotiana benthamiana* were successful after using hybrid receptors with the C-terminal part of ReMAX replaced by that of Eix2. These results show that ReMAX determines specificity for eMax. They also demonstrate hybrid receptor technology as a promising tool to overcome problems that impede interfamily transfer of PRRs to enhance pathogen detection in crop plants.

INTRODUCTION

As part of their innate immune systems, higher plants and animals have surface receptors, also referred to as pattern recognition receptors (PRRs), which recognize specific microbe-associated molecular patterns (MAMPs). In higher vertebrates, which also have an adaptive immune system, the PRR family includes a dozen Toll-like receptors and a few additional, structurally unrelated, receptors (Akira et al., 2006). Higher plants rely on innate immunity alone and seem to have vast arrays of PRRs, as suggested by the increasing number of different MAMPs that have been reported to trigger defense responses in various plant species (Boller and Felix, 2009). These molecular patterns include peptides, proteins, carbohydrates, lipids, and lipopolysaccharides (LPSs) that are typical for whole classes of microbes but do not occur in plant hosts.

Compared with the number of MAMPs, the number of PRRs with known ligand specificity is still small. Most of the these identified PRRs belong to the receptor-like kinase (RLK) or the receptor-like protein (RLP) families. Examples of defined ligand/receptor pairs of MAMPs and PRRs include flagellin/FLAGELLIN

SENSING2 (FLS2) (Gómez-Gómez and Boller, 2000), EF-Tu/EF-Tu RECEPTOR (EFR) (Zipfel et al., 2006), and peptidoglycan/LYSIN-MOTIF1 (LYM1) and LYM3 (Willmann et al., 2011) with MAMPs from bacteria as well as xylanase/ETHYLENE INDUCING XYLANASE2 (Eix2) (Ron and Avni, 2004), avirulence gene Ave1/VERTICILUM1 (Ve1) (de Jonge et al., 2012), and chitin/CHITIN ELICITOR RECEPTOR KINASE1 (Miya et al., 2007) with MAMPs from fungi, respectively.

MAMP perception in plants characteristically leads to a general state of resistance, also termed pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Early symptoms characteristically associated with MAMP perception include altered ion fluxes across the plasma membrane, leading to extracellular alkalization and increased Ca²⁺ concentration in the cytoplasm, induction of an oxidative burst, and enhanced biosynthesis of the stress hormone ethylene (Boller and Felix, 2009).

Many phytopathogenic microorganisms have evolved specific effector proteins that can block various steps of the plant defense response pathway (Jones and Dangl, 2006). In addition, some pathogens have evolved mechanisms to avoid immunodetection. For example, *Agrobacteria* and some species of *Xanthomonas* have changes in the flg22 epitope of flagellin that render this MAMP nondetectable by FLS2, the flagellin receptor of plants (Felix et al., 1999; Sun et al., 2006). In turn, recognition of a given type of microbe does not depend on a single MAMP. Apart from flagellin, *Arabidopsis thaliana* detects bacteria also via MAMPs like LPS (Dow et al., 2000), peptidoglycan (Gust et al., 2007), and EF-Tu (Kunze et al., 2004). Currently, the number of distinct perception systems with specificity for bacterial MAMPs is difficult to estimate. Identification of novel MAMPs and their corresponding PRRs remains an important issue to assess the repertoire of PRRs that collectively provide basal immunity to the plant.

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As a species, *Arabidopsis* is widespread in the northern hemisphere in Europe, central Asia, and North America where it is exposed to a broad range of environmental and climatic conditions (Hoffmann, 2002). More than 700 *Arabidopsis* accessions, previously also called ecotypes, have been collected from various habitats for studying natural variation of this plant species. As part of the 1001 *Arabidopsis* genome project (www.1001genomes.org), almost 500 of these accessions have been sequenced to date. Natural variation in these accessions also affects the repertoire of functional PRRs. For example, the accession Wassilewskija-0 carries a mutation in *FLS2* and is insensitive to flagellin (Gómez-Gómez et al., 1999; Zipfel et al., 2004). A more recent screen of 56 accessions revealed insensitivity to flagellin in three additional accessions as well as insensitivity to EF-Tu in two other accessions (Vetter et al., 2012). This opens the possibility that accessions with defects in the perception of other MAMPs might exist. Natural variation might thus be exploited as genetic tools to map and identify further PRRs.

Here, we report that accession Shakh dara (Sha), in contrast with many other accessions of *Arabidopsis*, lacks the ability to recognize eMAX, a proteinaceous MAMP occurring in xanthomonads. This allowed efficient mapping and identification of the corresponding PRR as a longer form of RECEPTOR-LIKE PROTEIN1 (RLP1), which we termed ReMAX (for receptor of eMax). The predicted structure of ReMAX/RLP1 resembles the tomato (*Solanum lycopersicum*) RLP Eix2, which detects fungal xylanase as a MAMP (Ron and Avni, 2004). A hybrid receptor with the ectodomain from ReMAX and the C-terminal part of Eix2 proved functional in eMAX perception when expressed in a non-native system like *Nicotiana benthamiana*. This identifies ReMAX as the PRR for eMax and suggests that a hybrid receptor strategy might prove useful for interfamily transfer of PRR function to enhance crop disease resistance.

RESULTS

Arabidopsis and Other Brassicaceae Detect a MAMP from *Xanthomonas*

To search for novel bacterial MAMPs and identify additional PRRs, we used an *Arabidopsis* double mutant for the two major known PRRs, *FLS2* and *EFR*; this double mutant line is unable to detect the flagellin and EF-Tu MAMPs. A cell culture system derived from these double mutant plants responded with a characteristic MAMP response, rapid extracellular alkalinization, when treated with preparations of *Xanthomonas axonopodis* pv *citri* strain 306 (*Xac*) (Figure 1A), indicating the presence of a MAMP that is not flagellin or EF-Tu. This response was triggered by treatment with intact bacteria, but responses were even stronger after sonication of the bacteria (Figure 1A). Further characterization showed that the eliciting activity was strongly reduced or abolished by heat treatment and digestion with proteases (Figure 1A), two features that distinguish the activity of *Xac* from peptidoglycan and LPS, two other, nonproteinaceous, bacterial MAMPs known to be recognized by *Arabidopsis* (Dow et al., 2000; Gust et al., 2007). A previous report

identified superoxide dismutase SodM from either *Xanthomonas campestris campestris* or *Escherichia coli* (Watt et al., 2006) as a MAMP recognized by cells of tobacco (*Nicotiana tabacum*; Watt et al., 2006). However, the *Arabidopsis* cells used in our study showed no response to SodM (see Supplemental Figure 1A online). In summary, these results indicate that the activity in extracts of *Xac* represents a proteinaceous MAMP that does not correspond to one of the previously identified MAMPs flagellin, EF-Tu, peptidoglycan, LPS, or SodM. An activity similar to the one observed in *Xac* could be detected in sonicated preparations obtained from other xanthomonads, including strains of *X. campestris* pv *vesicatoria* and *Xanthomonas arboricola* pv *juglandis* but not in extracts prepared from other bacteria like *Agrobacterium tumefaciens* or *Pseudomonas syringae* pv *tomato* (see Supplemental Figure 1B online).

The MAMP activity in the *Xac* extract was partially purified by anion exchange chromatography (Figure 1B). To monitor for MAMP activity, eluates from the column were assayed for induction of ethylene biosynthesis in leaf pieces of *fls2 efr* double mutant plants. Fractions with highest activity were combined, resulting in a preparation that induced half-maximal induction of ethylene biosynthesis at a concentration of ~ 1 μ g protein/mL (Figure 1C). This MAMP was termed eMax for “enigmatic MAMP of *Xanthomonas*” since, so far, our attempts to further purify and determine its molecular identity have not been successful.

Heat treatment abolished most of the MAMP activity of eMax when tested in wild-type Columbia-0 (Col-0) *Arabidopsis* (Figure 1D). This indicates that the single purification step via anion exchange chromatography resulted in a preparation essentially free of heat stable MAMPs such as the known bacterial MAMPs flagellin, EF-Tu, peptidoglycan, and LPS (Felix et al., 1999; Dow et al., 2000; Kunze et al., 2004; Gust et al., 2007). Testing eMax for induction of ethylene biosynthesis in various plant species revealed responsiveness in several species of the *Brassicaceae* family but not in tomato, *N. benthamiana*, pea (*Pisum sativum*), and other species belonging to different plant families (see Supplemental Figure 2 online).

MAMP perception in plants characteristically leads to a general state of resistance, also termed PTI (Jones and Dangl, 2006). *Arabidopsis* leaves pretreated with eMax similarly showed a significant increase in general resistance to infection with the bacterial pathogen *P. syringae* pv *tomato* and the fungal pathogen *Botrytis cinerea* (Figures 1E and 1F).

Responsiveness to eMax Maps to RLP1

In a screen including 61 different accessions of *Arabidopsis*, we detected that leaves from most of these accessions reacted to eMax with a significant increase in production of ethylene, as exemplified for the accessions Kondara and Tamm-27 in Figure 2 and summarized in Supplemental Table 1 online. Accession Sha was an exception and did not respond with increased ethylene biosynthesis when treated with eMax (Figure 2). Since Sha responded to other MAMPs like bacterial flg22 and the fungal extract Penicillium (Pen), it seemed specifically affected in the recognition of eMax.

Well-characterized recombinant inbred lines (RILs) of accession Sha with Landsberg *erecta* (*Ler*) (Clerkx et al., 2004) and

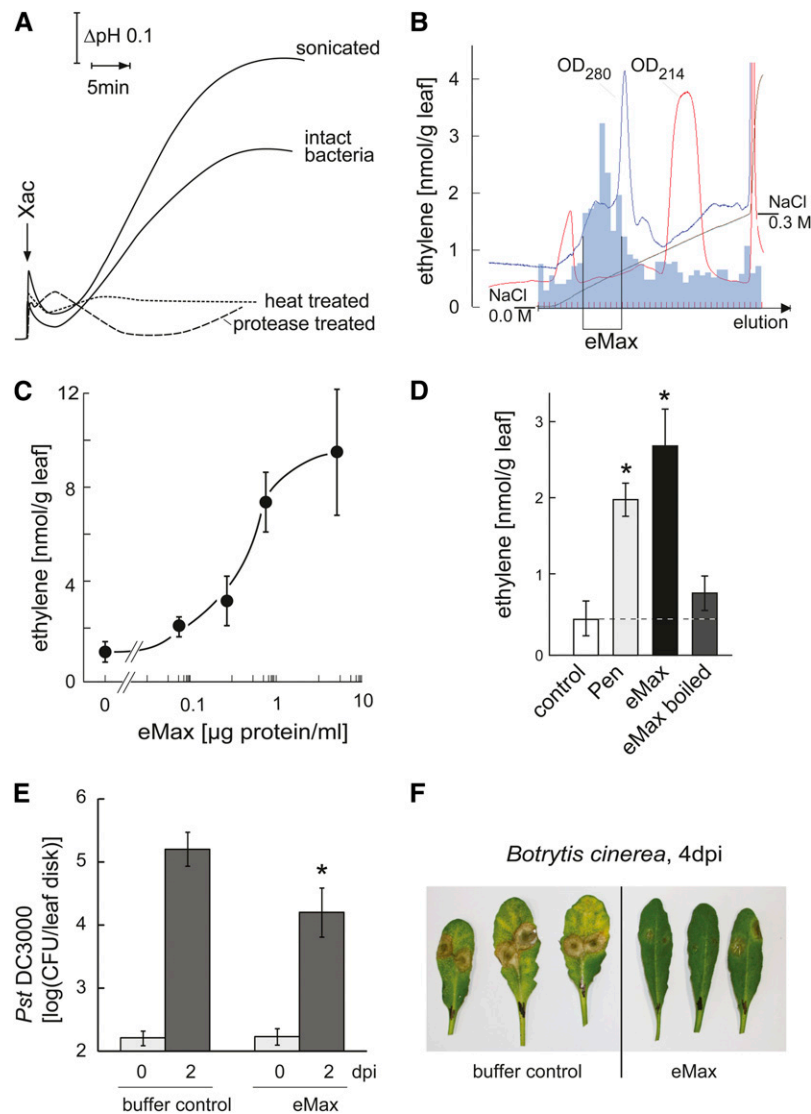


Figure 1. MAMP Activity from *Xac*.

(A) Preparations of living *Xac* bacteria or bacteria after sonication induce extracellular alkalinization in cultured cells of the *Arabidopsis* double mutant *fls2 efr* (Col-0 background). The activity is strongly impaired by heating (95°, 10 min) or by digestion with Proteinase K. The data show continuous pH tracings of representative examples from $n > 5$ repetitions of these experiments. The pH at the start of the experiment was 5.3, and scaling of time (x axis) and pH (y axis) were as indicated by the arrows.

(B) Partial purification of the activity in the *Xac* extract by anion exchange chromatography at pH 8.0. Eluate obtained by increasing NaCl concentration (x axis) was analyzed for OD₂₈₀ and OD₂₁₄. Fractions with highest activity (measured by ethylene emission from leaf pieces of *fls2 efr* plants; y axis) were pooled and denominated eMax as indicated.

(C) Dose dependency for ethylene induction. Leaf pieces of *fls2 efr* plants were treated with different concentrations of eMax. Values represent mean and SD of three replicates.

(D) Ethylene production of Col-0 leaf pieces treated with eMax (2 μ g/mL), heat-treated eMax (2 μ g/mL), or the fungal preparation Pen (90 μ g/mL) as positive control. Bars and error bars represent mean and SD of $n = 3$ replicates. Asterisks show results that differ significantly from control treatment (Student's *t* test, $P < 0.01$).

(E) PTI against *P. syringae* pv *tomato* strain DC3000 (*Pst* DC3000). *Arabidopsis fls2 efr* leaves were pretreated by pressure infiltration of leaves with 100 μ L of buffer (0.4 mM Tris, pH 8.0, with 5 mM NaCl) or buffer with eMax (0.6 μ g protein) for 12 h before pressure infiltration with *P. syringae* pv *tomato* strain DC3000. Bars and error bars show mean and SD of bacterial numbers (measured as colony-forming units [CFU], at the indicated days after infection [dpi]) from $n = 8$ replicates. Asterisks mark significant induction over control based on a Student's *t* test, P value < 0.01 .

(F) PTI against *B. cinerea*. *Arabidopsis fls2 efr* plants were sprayed with eMax or buffer and 12 h later infected by spotting 5 μ L drops containing ~ 500 spores of *B. cinerea*. Photographs were taken 4 d after infection.

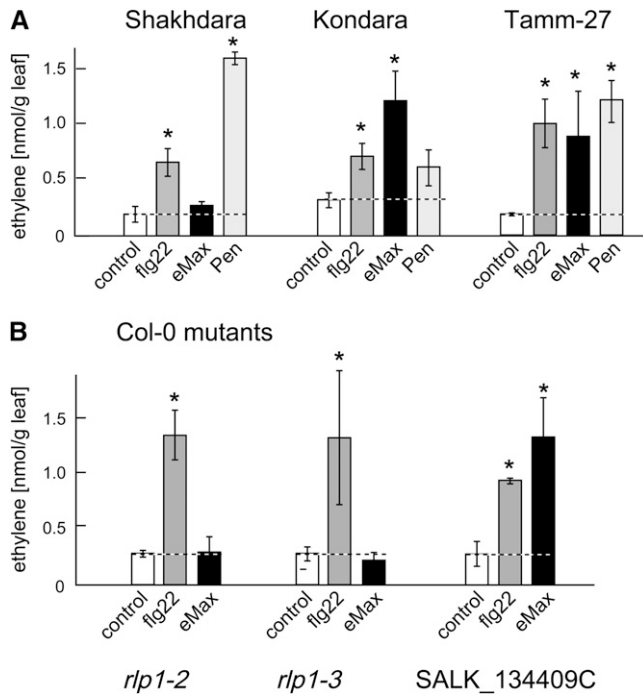


Figure 2. Response to eMax in Leaves from Different *Arabidopsis* Accessions and Mutants.

(A) Ethylene biosynthesis in response to eMax (2 $\mu\text{g}/\text{mL}$), flg22 (100 nM), and the fungal preparation Pen (90 $\mu\text{g}/\text{mL}$) in the accessions Sha, Kondara, and Tamm-27. Responses in the accessions Kondara and Tamm-27 are representative for the majority of the 61 accessions tested. **(B)** Response to eMax and flg22 in Col-0 plants with T-DNA insertions in the RLK gene At1g06840 (SALK_134409C) or the *RLP1* gene At1g07390, lines *rlp1-2* (SALK_116923) and *rlp1-3* (SALK_049403C), respectively. The bars and error bars show means and SD of three replicates. Asterisks mark significant induction over control based on a Student's *t* test, *P* value < 0.01.

Bayreuth (Bay-0) (Loudet et al., 2002) have been established and are available for mapping approaches. We tested the 114 RILs of *Ler* \times Sha collection (Clerkx et al., 2004) for ethylene biosynthesis in response to eMax and fungal Pen as a positive control. Only markers for the very top of chromosome 1 were linked with higher than 50% to responsiveness to eMax (see Supplemental Figure 3A online). The two additional markers T7I23 and T1G11 allowed to narrow the locus to a position between the markers T1G11 and F21M12.

For finer mapping, the 13 RILs from a cross between Bay-0 and Sha (Loudet et al., 2002; West et al., 2006) with recombinations between T1G11 and F21M12 were assayed for response to eMax. This allowed location of the trait to the 0.7 Mb between the genes At1g06460 and At1g08540 (see Supplemental Figure 3B online). This region comprises \sim 200 annotated genes in the reference genome, among them the two RLKs At1g06840 and At1g07650 and the RLP, At1g07390, respectively. We speculated that a defect in one of these receptor-type genes might cause insensitivity in Sha. The accession Sha is fully sequenced and can be compared with accessions that show responsiveness to eMax

(polymorph.weigelworld.org; Clark et al., 2007). According to this database, At1g07390 and At1g07650 display no single nucleotide polymorphisms (SNPs) that are unique for Sha and would lead to alterations in their amino acid sequences. At1g06840 has one SNP that causes a change from Val to Ile in its amino acid sequence. However, a mutant line with a T-DNA insertion in At1g06840 in the Col-0 background proved as responsive to eMax as the wild type (Figure 2B), excluding this gene from the list of candidates. Transcriptome data of Sha (West et al., 2006) revealed normal expression of At1g07650 but showed a strongly reduced transcription level for At1g07390 encoding RLP1. This was somewhat surprising since At1g07390 appeared to be free of SNPs also within >2 kb of its promoter region. We reexamined the genomic region encoding this gene in Sha and Col-0 and found a 7336-bp deletion in Sha covering the entire gene At1g07390 and also parts of the flanking genes At1g07380 and At1g07400 (see Supplemental Figure 4 online). A closer inspection of the polymorph database for the occurrence of deletions (SV Deletion; MPICao2010 version) indeed confirms this deletion in the Sha genome albeit only when sequences are searched region-wise rather than with the Atg numbers.

To corroborate the importance of *RLP1* for eMax perception, two independent T-DNA insertion mutants in At1g07390 of Col-0, *rlp1-2* and *rlp1-3*, were obtained. Like Sha, both mutant lines lacked responsiveness to eMax (Figure 2B).

A Version of *RLP1* Transcribed from an Upstream Start Site Encodes ReMAX

Three gene models with two distinct start sites have been proposed for *RLP1* (www.tair.de; Figure 3A). However, a closer inspection of the genomic sequence indicated that the open reading extends further to the 5' end, comprising a potential start codon 129 bp further upstream. We produced cDNA from Col-0 plants and demonstrated the presence of this longer transcript by amplifying with a primer encompassing this upstream start site. This cDNA predicts a longer form of RLP1, with nine exons encoding a protein of 1077 amino acids (Figure 3; see Supplemental Figure 5 online) that we tentatively termed ReMAX (for receptor of eMax).

ReMAX/RLP1 is a typical RLP (Jones et al., 1994) and resembles in its primary structure the well-characterized RLP Eix2 from tomato (Ron and Avni, 2004) (see Supplemental Figure 5 online). Eix2 detects fungal xylanase as a ligand and triggers typical MAMP responses, including induction of ethylene biosynthesis. Both RLPs have large extracellular domains with 32 and 31 LRRs, respectively, that are interrupted with island domains inserted before the last four of the LRRs. The LRR domains, flanked by characteristic pairs of Cys residues (Li and Chory, 1997), are followed by juxtamembrane domains with characteristic series of acidic residues, single membrane spanning transmembrane domains, and short cytoplasmic tails. While related with respect to the number of LRRs and the presence and position of island domains, the overall sequence identity between Eix2 and ReMAX/RLP1 is only \sim 29%. The proteins are most divergent in the amino acid residues that form the surface of LRR domain thought to be involved in ligand binding (see Supplemental Figure 5 online).

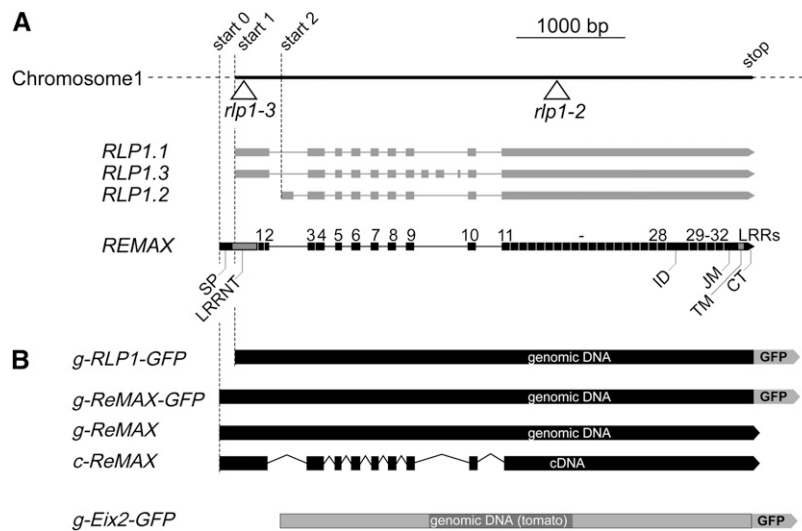


Figure 3. Gene Models for *RLP1/ReMAX* and Tomato *Eix2*.

(A) Three different gene models with start positions 1 at 2,269,893 or start 2 at 2,270,336 and different splicing options have been proposed for *RLP1/At1g07390* (www.tair.org). The mutant lines *rlp1-2* (SALK_116923) and *rlp1-3* (SALK_049403C) have T-DNA insertions in predicted exons as indicated. Sequencing of Col-0 cDNAs resulted in a gene model with a start at position 2,269,764 (start 0) that we define as *ReMAX*. SP, signal peptide; LRR-NT, N terminus of the LRR domain; ID, island domain; JM, juxtamembrane domain; TM, transmembrane domain; CT, cytoplasmic tail.

(B) Gene constructs used for complementation experiments: Genomic and cDNA with the start positions of *ReMAX* (start 0) and *RLP1.1/RLP1.3* (start 1) were cloned under the control of the 35S promoter. Where indicated, constructs were C-terminally fused in frame with a GFP tag. A similar construct with a GFP tag was produced for the RLP *Eix2* (Ron and Avni, 2004) based on genomic DNA from tomato.

ReMAX Restores eMax Response in *rlp1* Mutants of *Arabidopsis*

To confirm the role of *ReMAX*, different gene constructs were cloned from the genomic and cDNA, either with or without a C-terminal green fluorescent protein (GFP) tag, and used for complementation experiments (Figure 3B). Similarly, genomic DNA of tomato was used for a gene construct encoding the RLP *Eix2* (Ron and Avni, 2004) fused in frame to a C-terminal GFP tag (Figure 3B). These constructs were tested in complementation assays with mesophyll protoplasts from leaves of the mutants *rlp1-2*, *rlp1-3*, and *Sha* (Figure 4; see Supplemental Figure 6 online). For monitoring the MAMP response, protoplasts were cotransfected with *pFRK1:Luc*. This reporter construct with luciferase under the *FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1)* promoter was used for testing MAMP response in several prior studies (Asai et al., 2002; Yoo et al., 2007; Albert et al., 2010; Mueller et al., 2012). When transfected with the reporter *pFRK1:Luc* alone, protoplasts from *rlp1-2*, *rlp1-3*, and *Sha* showed no changes in luciferase activity following treatment with eMax; by contrast, protoplasts from wild-type Col-0 showed an increase in luciferase activity (Figure 4A; see Supplemental Figures 6B and 6D online). Similarly, no response to eMax was observed in protoplasts of *rlp1-2* after cotransformation of the reporter, and *RLP1-GFP* did not respond to eMax (Figure 4B). The *rlp1-2*, *rlp1-3*, and *Sha* protoplasts, however, gained responsiveness to eMax when transformed with the constructs *g-ReMAX*, *c-ReMAX*, or *g-ReMAX-GFP* that extend to the start codon on the full length cDNA (Figures 4C to 4E; see Supplemental Figures 6C and 6E online). These results demonstrated that *ReMAX* is indeed required

for eMax perception. They also show that functional *ReMAX* requires the transcriptional start site upstream of the start sites proposed for *RLP1*. Furthermore, the GFP tag fused to the short cytoplasmic tail of *ReMAX* seems not to compromise functionality of perception.

Interestingly, *Arabidopsis* protoplasts transformed with the construct encoding tomato *Eix2-GFP* showed reproducible and significant induction of the reporter gene after treatment with fungal xylanase (see Supplemental Figure 7 online). Although somewhat lower than the response to flg22 used as a positive control, this result showed that tomato *Eix2* can functionally interact with all elements required for transmembrane signaling and induction of downstream responses in cells of a plant from a different order.

A Hybrid RLP Made from *ReMAX* and *Eix2* Confers Perception of eMax to *N. benthamiana*

N. benthamiana lacks endogenous perception systems for xylanase and eMax (see Supplemental Figure 7 online). Confirming an earlier report (Ron and Avni, 2004), we observed that *N. benthamiana* leaves transformed with *Eix2-GFP* gain responsiveness to xylanase and respond with increased production of the stress hormone ethylene (see Supplemental Figure 8 online). By contrast, however, transfection of *g-ReMAX-GFP* was not sufficient to render *N. benthamiana* responsive to eMax. This could indicate that *ReMAX* alone does not form the genuine receptor site determining specificity for eMax. Alternatively, *ReMAX* might function as receptor site for eMax but encounter compatibility

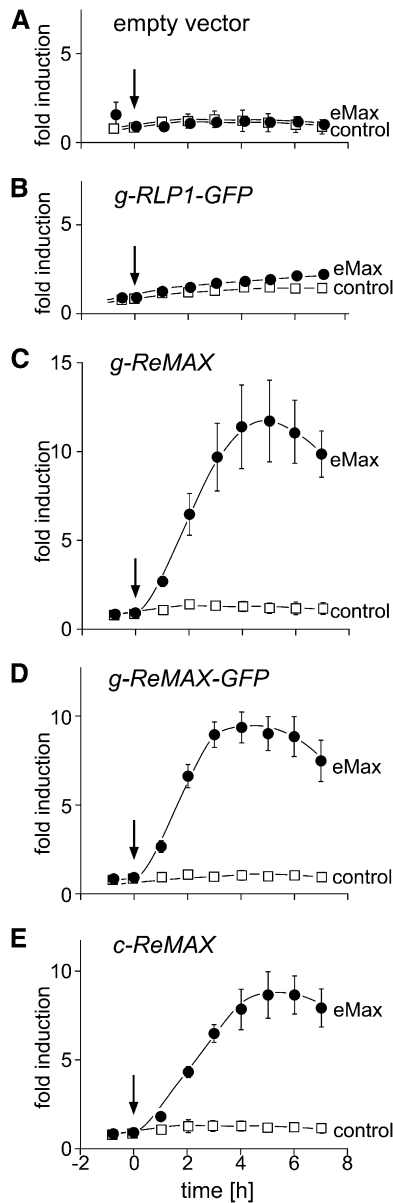


Figure 4. Perception of eMax in Protoplasts of *rlp1-2* (Col-0) Plants Expressing *RLP1/ReMAX* Constructs.

Protoplasts were cotransformed with the reporter construct *pFRK1:luc* and the different constructs of *RLP1/ReMAX* as indicated. At time $t = 0$, the protoplasts were treated with eMax (5 $\mu\text{g}/\text{mL}$) or with the inactive peptide *flg22^{A.tum}* (100 nM) as a control. Fold induction of luciferase activity was calculated with respect to values at time 0. Values, except for results in (B), represent means and SD of three replicates. Results shown are representative for $n \geq 3$ independent repetitions of the experiments.

problems with adaptor proteins required for receptor activation in the heterologous cell context of *N. benthamiana*. We addressed the second possibility by constructing chimeric forms of the RLPs ReMAX and Eix2 with defined, reciprocal, swaps of their C-terminal parts, as summarized in Figure 5A.

When expressed in *N. benthamiana* leaves, all constructs led to accumulation of tagged proteins with the expected size (see Supplemental Figure 9 online). Compared with Eix2, the construct $E^{\text{LRR-JM-R}}^{\text{TM-CT}}$, Eix2 with the transmembrane and C-terminal domains of ReMAX, lost much of its functionality as a xylanase receptor in *N. benthamiana* cells (Figure 5C), and the reciprocal construct $R^{\text{LRR-JM-E}}^{\text{TM-CT}}$ did gain some responsiveness to eMax (Figure 5B). However, in eight repetitions of this experiment responsiveness to eMax was either faint, 4 times, or not detectable, 4 times, respectively. Apparently, transmembrane and C-terminal domains of Eix2 are not sufficient for robust functionality in *N. benthamiana*. The results with the extended swaps, including also the juxtamembrane domain, were unequivocal and reproducible. Expression of $R^{\text{LRR-E}}^{\text{JM-TM-CT}}$ in *N. benthamiana* resulted in clear and significant response to eMax (Figure 5B) in all repetitions ($n > 6$). $E^{\text{LRR-R}}^{\text{JM-TM-CT}}$, in turn, was barely functional as xylanase receptor in *N. benthamiana* (Figure 5C). Thus, the C-terminal part including the apoplastic juxtamembrane domain determines functionality of ReMAX in different plant species. Equally important, this experiment also shows that the LRR ectodomain of ReMAX indeed specifies the specificity for the recognition of eMax, indicating that ReMAX is the bona fide receptor for eMax.

DISCUSSION

In this report, we started out from the observation that *Arabidopsis* has a sensitive detection system specific for a proteinaceous MAMP present in xanthomonads. MAMP activity of eMax is heat labile, indicating that the plant perception system is specific for the native configuration of the protein, similar to the perception of fungal xylanase by the tomato RLP Eix2 (Enkerli et al., 1999; Furman-Matarasso et al., 1999). Although remaining an important aim for future work, the molecular identification of the eMax protein was not a prerequisite to identify the corresponding PRR. A preparation essentially free of other types of MAMP was sufficient for a genetic approach to screen for genes involved in the perception of eMax. Identification of a particular PRR in plants can still present a big challenge, since these surface receptors occur in low abundance, rendering straightforward biochemical approaches difficult. Identification of most of the plant PRRs known to date relied on forward or reverse genetic approaches (Jones et al., 1994; Gómez-Gómez and Boller, 2000; Ron and Avni, 2004; Zipfel et al., 2006; Miya et al., 2007; Boller and Felix, 2009; Willmann et al., 2011; de Jonge et al., 2012), often starting out from natural variation observed between different varieties or strains of a species.

Natural variation with respect to presence or absence of particular PRRs is surprisingly high among different accessions of *Arabidopsis*. Accessions that lack either the PRR FLS2 for flagellin or the PRR EFR for EF-Tu have previously been reported (Gómez-Gómez et al., 1999; Vetter et al., 2012). In this work, we made use of this natural variation and screening of <70 accessions revealed a particular accession that was insensitive to eMax. Facilitated by the sets of genetic tools and comprehensive databases available for the model plant *Arabidopsis*, this perception system could be attributed to a particular member of the

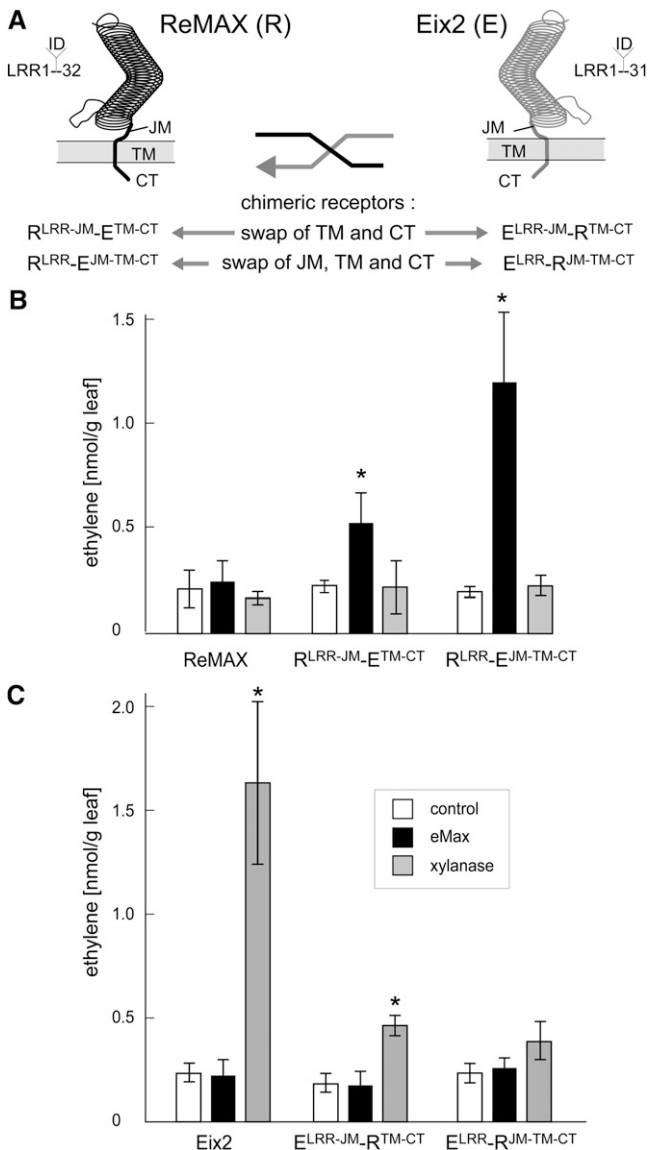


Figure 5. Functionality of ReMAX, Eix2, and Chimeric Forms of These RLPs in *N. benthamiana*.

(A) Schematic representation of ReMAX, Eix2, and the chimeric forms with reciprocal swaps of the C-terminal domains.

(B) and **(C)** Ethylene response of *N. benthamiana* transformed with the different constructs after treatment with Pen (90 μg/mL) as positive control, eMax (2 μg/mL), or xylanase (2 μg/mL) for 4 h. The bars show means and SD of three replicates. Asterisks mark significant induction over control based on a Student's *t* test, *P* value < 0.05.

RLP family. In *Arabidopsis*, the family of RLPs with apoplastic LRR domains comprises 57 members (Fritz-Laylin et al., 2005; Wang et al., 2010b). With the exceptions of the RLPs CLAVATA2 (CLV2; RLP10) and Too Many Mouths (TMM; RLP17), which play roles in developmental processes (Jeong et al., 1999; Nadeau and Sack, 2002), and RLP41 involved in abscisic acid-induced senescence (Wang et al., 2008), the functions of the other

members of this family remain unknown. Here, we show that ReMAX/RLP1 specifically functions as a PRR, sensing a specific proteinaceous signal occurring in *Xanthomonas*.

Lack of responsiveness to bacterial eMax in the accession Sha could be attributed to a deletion of >7 kb that includes the entire locus encoding RLP1. Independent mutant lines with T-DNA insertions in *RLP1* also lacked responsiveness to eMax, corroborating the importance of this gene for eMax perception. Restoring responsiveness to eMax in *rlp1* mutants and Sha plants required transfection with gene constructs encompassing a translational start site upstream of the ones proposed for *RLP1*. We termed this extended version of the RLP1 protein ReMAX.

The presence of intact ReMAX is a prerequisite for perception of eMax, but these genetic results cannot solve the question of whether ReMAX acts as the genuine receptor site for eMax. Without a clearly defined ligand at hand, interaction studies to demonstrate direct, specific interaction between eMax and ReMAX were not possible. Convincing evidence for the functionality and specificity of a receptor can also be obtained by expression in heterologous systems that lack an endogenous perception system for the particular ligand. Examples where this line of evidence successfully demonstrated receptor function include the *Arabidopsis* receptor kinase EFR expressed in *N. benthamiana* and tomato (Zipfel et al., 2006; Lacombe et al., 2010), the rice (*Oryza sativa*) receptor kinase XA21 expressed in *Citrus sinensis* (Cardoso et al., 2010), the tomato RLP Ve1 in *Arabidopsis* (Fradin et al., 2011). In our study, we found that Eix2 also functions in protoplasts of *Arabidopsis*, but *Arabidopsis* ReMAX, in turn, was nonfunctional as receptor of eMax in *N. benthamiana*. Functional perception of eMax was achieved by swapping the LRR domain of ReMAX to the C-terminal part of Eix2, thus demonstrating that the ectodomain of ReMAX was indeed determining specificity for the response to eMax (Figure 5).

Chimeric RLPs have previously been used to identify sites important for the ligand specificity of closely related Cf resistance proteins from tomato (van der Hoorn et al., 2005; Wulff et al., 2009). By contrast, in our study, chimeras of two RLPs exhibiting only 29% sequence identity were used to investigate subdomains important for downstream signaling in cells of heterologous plant species. Unlike receptor kinases, RLPs have only short cytoplasmic tails, and activation of downstream signaling is likely to depend on adaptors or coreceptors. A current theme for signaling via RLPs is heterodimerization with RLKs, as convincingly demonstrated for the RLP CLV2 and the receptor kinase CORYNE (Bleckmann et al., 2010; Wang et al., 2010a). The results with chimeric ReMAX and Eix2 suggests that the C-terminal part of these RLPs, including the extracellular juxta-membrane domain, the transmembrane domain, and the cytoplasmic tail, is important for interaction with adaptors and other receptor components essential for activation of downstream signaling. There is an apparent asymmetry between tomato Eix2, which functionally interacts with such components in cells of both *Arabidopsis* and *N. benthamiana*, and *Arabidopsis* ReMAX, which was not compatible in *N. benthamiana*. Chimeric forms also provide tools to further map the epitopes of RLPs

that are important for the compatibility with these adaptors and to investigate the molecular mechanism that leads to receptor activation in this class of PRRs.

The presence of *ReMAX/RLP1* appears to be restricted to species of *Brassicales*, and BLAST searches revealed no apparent orthologs in species outside this plant family. In turn, RLPs like Cf1 to Cf9, Ve1, and Eix2 found in *Solanales* have no functional counterpart in *Arabidopsis* (Fradin et al., 2011). More generally, apart from CLV2 and TMM, which are important for developmental processes common to all higher plants, all other RLPs seem not to be conserved among different plant families. RLPs with functions as PRRs seem to have evolved and diversified rather recently in evolution (Wang et al., 2010b) (e.g., by reduplication and shuffling of LRR subdomains). The genomic organization of *ReMAX/RLP1*, with several of the LRR subunits precisely separated by introns, might still reflect such a recent origin (Figure 3). The diversification of RLPs and RLKs in different species suggests that the variety of MAMPs detected by higher plants might greatly exceed the number of ligands currently known. Thus, species-specific repertoires of PRRs, including that of the model plant *Arabidopsis*, can serve as valuable resources to enlarge and enhance the immunodetection systems of agronomically important plants. This was successfully demonstrated recently for the receptor kinase EFR of *Arabidopsis*, which conferred increased resistance against bacteria to tomato (Lacombe et al., 2010).

In a very comprehensive survey, a collection of individual T-DNA insertions in the RLPs of *Arabidopsis* was assessed for changes in susceptibility to various microbial pathogens. This investigation also included infection with *X. campestris* pv *campestris* via application to wound sites or by infiltration into leaves. None of the knockout lines, including one of *RLP1/ReMAX*, showed a significant change in susceptibility to this pathogen (Wang et al., 2008). However, considering the redundancy of recognition systems, the loss of a single PRR might be limiting for plant defense only under specific conditions. Future experiments will have to include plants with multiple knockouts of PRRs for assessing the role of a particular RLP like *ReMAX*. Also, experimental conditions of infection are relevant, as observed for the flagellin receptor FLS2, which can restrict infection by *P. syringae* pv *tomato* after spray inoculation but not after pressure infiltration (Zipfel et al., 2004). Thus, more experiments with different strains of *Xanthomonas*, different initial doses and routes of pathogen application, as well as different growth conditions of the host plants will be required to assess the function of *ReMAX* for host defense. Alternatively, using chimeric forms of *ReMAX* as described in this article, it will be possible to test gain of eMax perception for resistance against xanthomonads in *Solanaceous* plants.

In summary, in this study, we could attribute *ReMAX* of *Arabidopsis* with the specific perception of a MAMP from *Xanthomonas*. Chimeric constructs that combine functional elements from the RLPs *ReMAX* and *Eix2* demonstrate that *ReMAX* acts as the genuine receptor for eMax. This chimeric approach provides a new option for transferring functional PRRs to rather distantly related plants, thus allowing researchers to equip agronomically important crop species with novel specificities for pathogen recognition.

METHODS

Materials

Xylanase from *Trichoderma viride* (Sigma-Aldrich) was purified by ion-exchange chromatography as described (Enkerli et al., 1999). The fungal preparation Pen from *Penicillium chrysogenum* (Thuerig et al., 2005) and the peptides flg22 and flg22^{A.tum} (Felix et al., 1999) were used as described before. *Xanthomonas* strains used were *Xanthomonas axonopodis* pv *citri* strain 306 (*Xac*) (da Silva et al., 2002), *Xanthomonas arboricola* pv *juglandis* strain DSM-1049 (*Xaj*) (DSMZ), and *Xanthomonas campestris* pv *vesicatoria* strain 85-10 Bonas et al., 1989).

Plant Material

Arabidopsis thaliana accessions, the RILs of *Ler* × *Sha* and *Bay* × *Sha*, the lines SALK_116923 (*rlp1-2*), SALK_049403C (*rlp1-3*), and SALK_134409C (T-DNA insertion in At1g06840) were from the Nottingham Arabidopsis Stock Centre. The double mutant *fls2 efr* (SAIL_691C4 × SALK_044334) was obtained from V. Nekrasov (Nekrasov et al., 2009).

Medium Alkalinization and Ethylene Measurement

Medium alkalinization in suspension cultured cells and ethylene biosynthesis in leaf tissue as assays for MAMP responses were performed as described before (Felix et al., 1999). To test for general responsiveness to MAMPs preparations of Pen (Thuerig et al., 2005), elf18 (Zipfel et al., 2006) and flg22 were used as positive controls and flg22^{A.tum} as negative control, respectively (Felix et al., 1999).

Preparation of eMax

X. axonopodis pv *citri* was grown on Kings B plates for 48 h at 30°C. The bacteria were harvested and sonicated three times for 2 min (50 W; SONOPULS HD UW2070; Bandelin). Supernatant was dialyzed (molecular weight cut off: 4 to 6 kD; Roth) and separated by anion exchange chromatography (Tris-HCl, pH 8.0; Q-Sepharose; GE Healthcare). Fractions with highest activity as inducer of ethylene biosynthesis in the double mutant *fls2 efr* were pooled and termed eMax.

PTI

For testing effects on growth of *Pseudomonas syringae* pv *tomato* strain DC3000 (Katagiri et al., 2002), *Arabidopsis* leaves were pretreated for 12 h by injection with 100 µL of buffer (0.4 mM Tris, pH 8.0, containing 5 mM NaCl) or buffer with eMax (0.6 µg protein). Bacteria in 10 mM MgCl₂ were applied at a density of 10⁵ colony-forming units mL⁻¹ (OD₆₀₀ = 0.002) and colony-forming units in extracts of leaves determined 1, 24, and 48 h later.

For assaying the effect on growth of *Botrytis cinerea* BO5-10 (Mengiste et al., 2003), leaves were sprayed with buffer (0.4 mM Tris, pH 8.0, containing 5 mM NaCl) or eMax in buffer (6 µg protein mL⁻¹). After 12 h of pretreatment, leaves were infected by spotting 5-µL drops of a suspension containing 10⁵ conidia mL⁻¹. Plants were incubated at 100% relative humidity at 22°C under short-day conditions, and appearance of visual symptoms was monitored over 4 d.

Cloning of Receptor Constructs

All PCRs were performed with the Phusion Hot Start DNA Polymerase (Fermentas), and constructs were cloned via *Sma*I and *Bam*HI sites into vectors derived from pPGT, a derivative of pZP212 (Hajdukiewicz et al., 1994) that contains the cauliflower mosaic virus 35S promoter, the GFP5

(S65T) coding region (Friedrichsen et al., 2000), and the *rbcS* terminator (Hajdukiewicz et al., 1994). Where indicated, receptor constructs were C-terminally fused to a GFP tag. PCR was based on genomic DNA or cDNA with primers listed in Supplemental Figure 9 online. Chimeric constructs were generated by fusion of two separate PCR products as described (Albert et al., 2010) with the primers indicated in Supplemental Figure 10 online.

Expression in *Nicotiana benthamiana* Leaves

Agrobacterium tumefaciens carrying plasmids encoding the gene for expression were mixed 1:1 with *A. tumefaciens* harboring the p19 suppressor of silencing and pressure infiltrated into leaves of 4- to 5 week-old *N. benthamiana* as described (Voinnet et al., 2003). Leaves were cut and used for bioassays at 24 to 48 h after infiltration or extracted for immunoblot analysis at 48 to 72 h after infiltration, respectively.

Expression and Functionality Assay in *Arabidopsis* Mesophyll Protoplasts

Transient expression in leaf mesophyll protoplasts was performed as described (Yoo et al., 2007). Aliquots of 80,000 protoplasts were co-transformed with 5 µg of plasmid DNA encoding firefly luciferase under the *FRK1* promoter (*pFRK1:luc*) (Asai et al., 2002) and 20 µg of plasmid DNA encoding the receptor construct to be tested. The protoplasts were re-suspended in W5 solution supplemented with 200 µM firefly luciferin and distributed to wells in a 96-well plate (10,000 protoplasts/well). After 16 h of preincubation, protoplasts were assayed for response to MAMPs by measuring luminescence of protoplasts using a luminometer (Mithras LB 940; Berthold).

Mapping a Locus Important for eMax Perception

The collection of 114 RILs from *Ler* Sha (Clerkx et al., 2004) was tested for response to eMax. The 24 RILs with recombinations between the markers NGA59, F21M12, and F3F19 on top of chromosome 1 were analyzed with the two additional AFLP (amplified fragment length polymorphism) markers T7I23 and T1G11 (www.inra.fr/internet/Produits/vast/msat.php). For further analysis, an additional 13 RILs from a cross between Bay-0 and Sha (Loudet et al., 2002; West et al., 2006) with mapped recombinations between T1G11 and F21M12 were used.

Accession Numbers

Sequence information can be found in the GenBank/EMBL or Arabidopsis Genome Initiative databases for RLP1 (At1g07390) and at the Sol Genomics Network for Eix2 (Solyc07g008630.1).

Supplemental Data

The following materials are available in the online version of this article.

- Supplemental Figure 1.** Extracellular Alkalinization in *fls2 efr* Cells.
- Supplemental Figure 2.** Response to eMax in Leaves of Different Plant Species.
- Supplemental Figure 3.** Responsiveness to eMax Maps to Top of Chromosome 1.
- Supplemental Figure 4.** The accession Shakhara has a 7336 bp deletion on chromosome 1.
- Supplemental Figure 5.** Primary Sequence of ReMAX and the Xylanase Receptor Eix2.
- Supplemental Figure 6.** Complementation of the *rlp1-3* Mutant and Sha with *g-ReMAX*.

Supplemental Figure 7. Eix2 Functions as Xylanase Receptor in *Arabidopsis* Protoplasts.

Supplemental Figure 8. ReMAX and Eix2 After Heterologous Expression in *N. benthamiana*.

Supplemental Figure 9. Expression of ReMAX, Eix2 and Chimeric Receptor Forms in *N. benthamiana* Leaves.

Supplemental Figure 10. Primers Used in This Study.

Supplemental Table 1. MAMP Response in 61 Accessions of *Arabidopsis*.

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AUTHOR CONTRIBUTIONS

A.K.J., M.L., M.A., V.F.-M., U.F., and K.M. performed research and analyzed data. A.K.J., M.L., and G.F. designed the research and wrote the article.

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