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Perception of the novel MAMP eMax from different *Xanthomonas* species requires the *Arabidopsis* receptor-like protein ReMAX and the receptor kinase SOBIR

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As part of their innate immune system plants carry a number of pattern recognition receptors (PRRs) that can detect a broad range of microbe-associated molecular patterns (MAMPs). In a recently published article[1] we described a novel, proteinaceous MAMP termed eMax (enigmatic MAMP of *Xanthomonas*) that derives from *Xanthomonas* and gets recognized by the receptor-like protein ReMAX (RECEPTOR OF eMax) of *Arabidopsis thaliana*. ReMAX has no ortholog in *Nicotiana benthamiana* and this species does not respond to eMax even when transformed with ReMAX. However, interfamily transfer of eMax perception was successful with a chimeric form of ReMAX where the C-terminal part of the protein was replaced by the corresponding part of the tomato RLP EIX2 (ETHYLENE INDUCING XYLANASE2). In this addendum we describe the difficulties with the purification and identification of the MAMP eMax and we present data demonstrating that functionality of ReMAX, much like that of related RLPs, depends on the presence of the receptor kinase SOBIR (SUPPRESSOR OF BIR1–1).

Higher plants carry pattern recognition receptors (PRRs) on their surface, which recognize microbe-associated molecular patterns (MAMPs). These MAMPs are typical for whole classes of microbes but do not occur in plant hosts.[2] Prominent bacterial MAMPs are flagellin and the Elongation Factor Tu (EF-Tu), where the model plant *Arabidopsis thaliana* recognizes the peptides flg22 and elf18[3,4] as minimal epitopes. Most identified PRRs, such as the receptors FLS2 for bacterial flagellin, EFR for bacterial EF-Tu or EIX2 for fungal xylanase,[5-7] belong to the receptor-like kinase (RLK) or the receptor-like protein (RLP) families. Early symptoms of MAMP perception include altered ion fluxes across the plasma membrane, leading to extracellular alkalinization and Ca^{2+} influx, ROS (reactive oxygen species) production and enhanced ethylene biosynthesis[8] inducing a general state of resistance (pattern-triggered immunity (PTI).[9] To avoid immune reactions of the plant host, pathogens have evolved different strategies: Some pathogens produce effector proteins influencing the plant defense response pathway.[9] Another strategy is the avoidance of recognition by altering the recognized epitope, as shown for *Agrobacteria* and some species of *Xanthomonas*, which are modified in the flg22 epitope of flagellin.[4,10] However, it is noteworthy that pathogen recognition in plants is based on multiple, redundant perception systems detecting several MAMPs of a pathogen. Many MAMPs and PRRs are yet not identified and thus newly discovered MAMP-PRR pairs are essential for a better understanding of the plant innate immunity. The repertoire of the PRRs varies not only between plant species but also between different accessions of *A. thaliana*. Examples are the receptors FLS2 and EFR, which are altered and thus not functional in some of the accessions.[11-13] In our recently published article, we used this natural variation to identify the PRR of the novel MAMP eMax.[1]

The study was initiated by the observation that extracts of *Xanthomonas axonopodis* pv *citri* induce ethylene production and extracellular alkalinization in *Arabidopsis fls2 efr* mutants that are not able to detect the well-known bacterial MAMPs flagellin and EF-Tu. The novel MAMP activity can be solubilized from the bacteria by sonication, is heat labile, and protease sensitive. The protein, termed enigmatic MAMP of *Xanthomonas* (eMax), binds to anion exchange columns and can be eluted with low salt concentrations. Obviously, this single purification step is unlikely to result in a preparation containing pure eMax alone. But is it sufficient to separate eMax from other bacterial MAMPs? For initial characterization of the activity from *Xanthomonas* interference by the MAMPs flagellin and EF-Tu was excluded by using the *fls2 efr* double mutant. Working with wild-type plants, in turn, relies on the absence of known MAMPs like flagellin, EF-Tu, peptidoglycan

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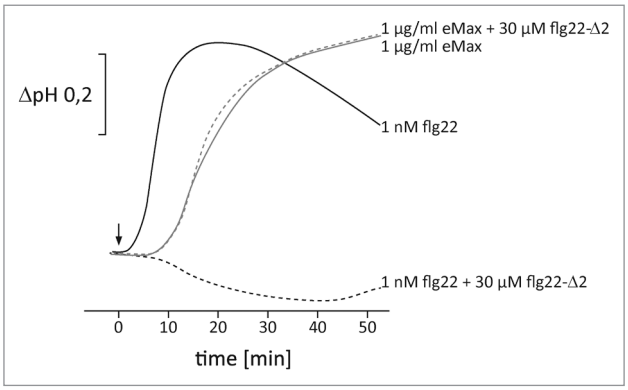

Figure 1. Testing eMax for contamination with flagellin. Extracellular alkalinization in cell cultures of *A. thaliana* wild-type cells to test the response to flg22 and eMax in the presence or absence of the flagellin antagonist flg22-Δ2.

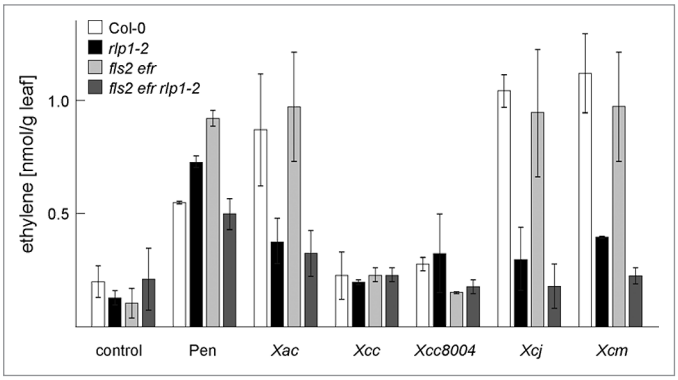

Figure 2. Ethylene biosynthesis in response to different Xanthomonas strains. Col-0 wild-type and the mutants *rlp1–2*, *fls2 efr* and *fls2 efr rlp1–2* were treated with extracts of different Xanthomonas strains and the fungal extract Pen (90 µg/ml)[18] as positive control (*Xac*: *Xanthomonas axonopodis* pv *citri*, *Xcc*: *Xanthomonas campestris* pv *campestris*, *Xcj*: *Xanthomonas campestris* pv *juglandis*, *Xcm*: *Xanthomonas campestris* pv *malvacearum*). The bars and error bars represent means and standard deviations of three replicates.

and LPS that are usually present in crude bacterial extracts and could interfere in the assays. These known MAMPs are all heat stable while, in contrast, the activity of the eMax preparation was found to strongly decrease after boiling for 5 min. Heat lability of eMax thus served as a first criterion to distinguish the novel activity from other MAMPs known to be perceived by *Arabidopsis*. For the highly abundant bacterial MAMPs EF-Tu and flagellin there are also additional tests with antagonistic peptides[6,14] that interrupt downstream signaling. For example, flg22-Δ2, a C-terminally truncated version of flg22, acts as a specific antagonist for flagellin in *Arabidopsis thaliana*.[14] Addition of excess flg22-Δ2 to *Arabidopsis* wild-type cell cultures blocks the response to subsequent treatment with non-saturating doses of flg22 while, in contrast, this pretreatment had no effect on the response to the eMax preparation, indicating that flagellin is not responsible for the response observed within these fractions (**Fig. 1**). Confirming absence of flagellin, several species outside the family of *Brassicaceae* that do respond to flg22 showed no response when treated with the eMax preparation.[1]

However, this does not mean that eMax is the only protein in the preparation and analysis by SDS-PAGE reveals that this preparation is heterogenous and contains different proteins. Additional attempts of purification using a variety of other chromatographic methods including reversed phase chromatography, cation exchange chromatography and separation by isoelectric focusing, was either not successful due to irreversible loss of eMax or resulted in purified fractions containing significant MAMP activity but insufficient amounts of protein for MS analysis. This indicates that eMax is active at very low concentrations, a feature that might render its identification a tricky task.

The partially purified preparation of eMax, devoid of other known MAMPs, was used to screen for natural variation of eMax perception in a collection of *Arabidopsis* accessions from different geographical origins. This allowed the identification of the accession Shakhdara (Sha) as insensitive to eMax. Recombinant inbred lines between the insensitive accession Sha and the sensitive accessions L*er* or Bay-0 allowed mapping of the locus responsible for sensing eMax to the RECEPTOR-LIKE PROTEIN 1, (At1g07390) that we termed ReMAX for RECEPTOR OF eMax. Insertional knockout mutants of *remax/rlp1* in the Col-0 background lack responsiveness to the eMax preparation. This also provides clear evidence that the eMax preparation used in our study contains only one predominant type of MAMP that is perceived by the single receptor ReMAX in *Arabidopsis*.

Arabidopsis mutants lacking RLP1/ReMAX will be of use for further characterization of eMax but also for studying the role of this perception system for plant immunity. By crossing the double mutant *fls2 efr* with *remax/rlp1*-2 we obtained the triple mutant *fls2 efr rlp1–2* as an additional genetic tool. Comparing the induction of ethylene biosynthesis in leaves from *fls2 efr* and *fls2 efr rlp1–2* showed that eMax is present in extracts from *Xanthomonas axonopodis* pv *citri*, *X. campestris* pv *juglandis*, *X. campestris* pv *malvacearum* but no or only little activity in extracts from the two strains of *X. campestris* pv *campestris* (**Fig. 2**). Thus, tests with additional *Xcc* strains will be required to learn more about the occurrence of eMax, which may also help to identify eMax.

One explanation for this apparent lack of eMax in *Xcc* might be that this pathovar is adapted to *Brassicaceae*. Thus, eMax might be absent or modified in *Xcc*, rendering this pathogen less detectable by ReMAX. Modification of MAMPs that reduce recognition by the corresponding pattern recognition receptors in different host plants has been shown for flagellin of *Agrobacteria* and *Xanthomonas*.[4,10] Whether such a strategy to avoid recognition also applies to *Xcc* will require the identification of eMax. Another possibility might be that these strains contain inhibitors that suppress MAMP recognition or downstream signaling. However, these two options should not be seen as mutually exclusive and remain to be tested experimentally.

Heterologous expression of authentic ReMAX in *N. benthamiana* was not sufficient to confer responsiveness to eMax. However, complementation of the *Arabidopsis rlp1* mutants with ReMAX was successful. Thus, chimeric receptors were designed using the

coding sequences of ReMAX and the structurally related receptor-like protein EIX2 from tomato that detects fungal xylanase.[7] An artificial receptor consisting of the complete ReMAX LRR-domain and the C-terminal part of EIX2 with its outer juxtamembrane domain was able to transfer the capacity for eMax perception into *N. benthamiana*. This indicates that the *Arabidopsis* ReMAX receptor might be incompatible with *N. benthamiana* orthologs of co-receptors or adaptor proteins that are required for receptor functionality. Functionality of RLPs indeed seems to depend on the receptor kinase SOBIR (SUPPRESSOR OF BIR1–1) as an adaptor protein. This was first described for RLPs of tomato[15] and, more recently, also for the functionality of *Arabidopsis* RLP30 as a PRR for the novel MAMP SCFE1 secreted by the fungus *Sclerotinia sclerotiorum*.[16] We found that *sobir* mutants also lacked responsiveness to eMax (**Fig. 3**). This result corroborates the hypothesis that SOBIR might be generally required as an adaptor protein for RLPs with functions as PRRs.[17] Further experiments will now be required to elucidate the role of SOBIR for the activation of RLP-type of receptors.

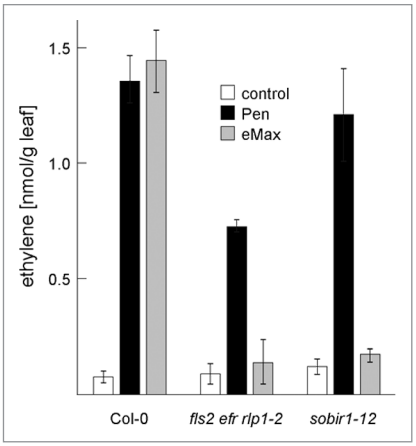

Figure 3. *sobir* mutants lack responsiveness to eMax. Ethylene biosynthesis in response to eMax (2 µg/ml) and Pen (90 µg/ml)[18] in leaves from Col-0 wild-type and the mutants *fls2 efr rlp1–2* and *sobir1–12* (SALK_050715[19]). The bars and error bars represent means and standard deviations of three replicates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Jehle AK, Lipschis M, Albert M, Fallahzadeh-Mamaghani V, Fürst U, Mueller K, Felix G. The receptor-like protein ReMAX of Arabidopsis detects the microbe-associated molecular pattern eMax from Xanthomonas. Plant Cell 2013; 25:2330-40; PMID:23898033; http://dx.doi.org/10.1105/tpc.113.110833
2. Zipfel C. Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol 2008; 20:10-6; PMID:18206360; http://dx.doi.org/10.1016/j.coi.2007.11.003
3. Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G. The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 2004; 16:3496-507; PMID:15548740; http://dx.doi.org/10.1105/tpc.104.026765
4. Felix G, Duran JD, Volko S, Boller T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 1999; 18:265-76; PMID:10377992; http://dx.doi.org/10.1046/j.1365-313X.1999.00265.x
5. Gómez-Gómez L, Boller T. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell 2000; 5:1003-11; PMID:10911994; http://dx.doi.org/10.1016/S1097-2765(00)80265-8
6. Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 2006; 125:749-60; PMID:16713565; http://dx.doi.org/10.1016/j.cell.2006.03.037
7. Ron M, Avni A. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. Plant Cell 2004; 16:1604-15; PMID:15155877; http://dx.doi.org/10.1105/tpc.022475
8. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 2009; 60:379-406; PMID:19400727; http://dx.doi.org/10.1146/annurev.arplant.57.032905.105346
9. Jones JD, Dangl JL. The plant immune system. Nature 2006; 444:323-9; PMID:17108957; http://dx.doi.org/10.1038/nature05286
10. Sun W, Dunning FM, Pfund C, Weingarten R, Bent AF. Within-species flagellin polymorphism in Xanthomonas campestris pv campestris and its impact on elicitation of Arabidopsis FLAGELLIN SENSING2-dependent defenses. Plant Cell 2006; 18:764-79; PMID:16461584; http://dx.doi.org/10.1105/tpc.105.037648
11. Vetter MM, Kronholm I, He F, Häweker H, Reymond M, Bergelson J, Robatzek S, de Meaux J. Flagellin perception varies quantitatively in Arabidopsis thaliana and its relatives. Mol Biol Evol 2012; 29:1655-67; PMID:22319159; http://dx.doi.org/10.1093/molbev/mss011
12. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T. Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 2004; 428:764-7; PMID:15085136; http://dx.doi.org/10.1038/nature02485
13. Gómez-Gómez L, Felix G, Boller T. A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J 1999; 18:277-84; PMID:10377993; http://dx.doi.org/10.1046/j.1365-313X.1999.00451.x
14. Bauer Z, Gómez-Gómez L, Boller T, Felix G. Sensitivity of different ecotypes and mutants of Arabidopsis thaliana toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J Biol Chem 2001; 276:45669-76; PMID:11564731; http://dx.doi.org/10.1074/jbc.M102390200
15. Liebrand TW, van den Berg GC, Zhang Z, Smit P, Cordewener JH, America AH, Sklenar J, Jones AM, Tameling WI, Robatzek S, et al. Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. Proc Natl Acad Sci U S A 2013; 110:10010-5; PMID:23716655; http://dx.doi.org/10.1073/pnas.1220015110
16. Zhang W, Fraiture M, Kolb D, Löffelhardt B, Desaki Y, Boutrot FF, Tör M, Zipfel C, Gust AA, Brunner F. Arabidopsis RECEPTOR-LIKE PROTEIN30 and Receptor-Like Kinase SUPPRESSOR OF BIR1-1/EVERSHED Mediate Innate Immunity to Necrotrophic Fungi. Plant Cell 2013; 25:4227-41; PMID:24104566; http://dx.doi.org/10.1105/tpc.113.117010
17. Liebrand TW, van den Burg HA, Joosten MH. Two for all: receptor-associated kinases SOBIR1 and BAK1. Trends Plant Sci 2013; PMID:24238702; http://dx.doi.org/10.1016/j.tplants.2013.10.003
18. Thuerig B, Felix G, Binder A, Boller T, Tamm L. An extract of *Penicillium chrysogenum* elicits early defense-related responses and induces resistance in *Arabidopsis thaliana* independently of known signalling pathways. Physiol Mol Plant Pathol 2006; 67:180-93; http://dx.doi.org/10.1016/j.pmpp.2006.01.002
19. Gao M, Wang X, Wang D, Xu F, Ding X, Zhang Z, Bi D, Cheng YT, Chen S, Li X, et al. Regulation of cell death and innate immunity by two receptor-like kinases in Arabidopsis. Cell Host Microbe 2009; 6:34-44; PMID:19616764; http://dx.doi.org/10.1016/j.chom.2009.05.019