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The *Arabidopsis* immune receptor EFR increases resistance to the bacterial pathogens *Xanthomonas* and *Xylella* in transgenic sweet orange

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Citrus agribusiness faces major economic losses due to bacterial diseases (Caserta *et al.*, 2020). Citrus canker (CC) and citrus variegated chlorosis (CVC) caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) and *Xylella fastidiosa* subsp. *pauca* (*Xfp*), respectively, are important threats in commercial citrus orchards. All sweet orange (*Citrus sinensis*) commercial varieties are susceptible to both pathogens, and no natural resistance has been found so far.

Plant cell-surface receptors recognize pathogen (or microbe)-associated molecular patterns (PAMPs/MAMPs) to activate pattern-triggered immunity (PTI). The well-studied *Arabidopsis thaliana* ELONGATION FACTOR-TU RECEPTOR (EFR) recognizes the conserved bacterial PAMP EF-Tu and derived elf peptides (Boutrot and Zipfel, 2017). Interfamily transfer of EFR has been shown to increase anti-bacterial disease resistance in several crops. The manipulation of PTI-related genetic traits has the potential to create more durable resistance assuring a sustainable productivity (Boutrot and Zipfel, 2017).

EF-Tu is present in the biofilm of both bacteria (Silva *et al.*, 2011; Zimaro *et al.*, 2013) and the outer membrane vesicles (OMVs) released by *X. fastidiosa* (Feitosa-Junior *et al.*, 2019). We hypothesized that *EFR* gene transfer is a powerful strategy to increase *Citrus* broad-spectrum resistance against CC and CVC. We first used the genetic models *Arabidopsis* and *Nicotiana tabacum* to address whether EFR can recognize these bacteria. *Arabidopsis* Col-0 (wild-type, WT) produced reactive oxygen species (ROS) when challenged with living *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*), which was markedly reduced in *efr-1* mutants (Fig. 1a). Similarly, *Arabidopsis* seedling growth was strongly repressed in the presence of *Xff* OMVs (Fig. 1b). No growth repression was observed in *efr-1* or *bak1-5*, a mutant affected in the EFR co-receptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Schwessinger *et al.*, 2011). We then examined whether EFR modulates *Xff* infection in *Arabidopsis* (Pereira *et al.*, 2019). Compared to Col-0, *efr-1* supported higher bacterial loads, quantified as *HL5/HL6* abundance (Fig. 1c). These observations suggest the effective perception of *Xff* EF-Tu by EFR, showing that it is sufficient to restrict *Xff* colonization in *Arabidopsis*.

We next investigated whether *Xfp*- and *Xcc*-derived elf peptides activate EFR-dependent immune responses. We tested ROS production in Col-0 and transgenic tobacco expressing *EFR*, both showing ROS production quickly after $elf18_{Ec}$ (*E. coli*) exposure. Although there are sequence differences between the elf peptides (Fig. 1g), ROS was similarly produced in Col-0 after $elf18_{Xcc}$ or $elf26_{Xfp}$ treatment, albeit to a lower extent in the later case (Fig. 1d). A similar pattern was observed for transgenic tobacco expressing *EFR* (Fig. 1e). Together, these findings indicate that EFR recognize EF-Tu from citrus phytobacteria,

indicating that its transfer to sweet orange might be a good strategy to confer broad-spectrum recognition.

To test whether EFR is functional in citrus, nine independent transgenic lines of Valencia sweet orange (V1 to V9) expressing *EFR* were obtained. Transgene integration and expression were confirmed through histochemical GUS assay and RT-qPCR, respectively (Fig. 1f). Each line had a single transgene copy. Seedlings were grafted in Rangpur lime rootstocks, showing normal development. Transgenic leaf discs challenged with $elf18_{Ec}$, $elf18_{Xcc}$ or $elf26_{Xfp}$ showed variable levels of ROS production depending on the peptide (Fig. 1h). Treatment with $elf18_{Ec}$ and $elf18_{Xcc}$ produced similar results, although slightly delayed for $elf18_{Xcc}$. The lines V4 and V5 showed responsiveness to $elf26_{Xfp}$ but at relatively low level (Fig 1h). The elf-induced ROS production in EFR transgenic citrus indicates functional conservation of the required intracellular signaling components in sweet orange.

Since V4 and V5 responded to the three peptides, they were further tested for the activation of mitogen-activated kinases (MAPK) and expression of defense genes. MAPK phosphorylation was detected in both lines compared to the mock treatment 30 and 45 minutes after elf treatment, with stronger signal after 45 minutes (Fig. 1i). Interestingly, constitutive activation was observed in the V5 line; yet the signal further increased after peptide treatment. The citrus defense genes *SGT1*, *EDS1*, *WRKY23,* and *NPR2* (Shi *et al.*, 2015) revealed that all genes were induced 3 hours after peptide treatment at different extents in the transgenic lines compared to respective mock samples (Fig. 1j). Defense gene upregulation in response to most peptides was stronger in V5 line. A stronger induction was triggered after $elf18_{Ec}$ and $elf18_{Xcc}$ compared to $elf26_{Xfp}$ treatment (Fig. 1j), following the patterns observed for ROS production.

Next, we evaluated whether the transgenic plants show enhanced resistance to CC and CVC. Detached leaves were infiltrated with the *Xcc* strain 306 bacterial suspension (10^4 CFU mL^{-1}) expressing GFP (Rigano *et al.*, 2007). Canker lesions developed in all inoculated leaves 14 days after inoculation (dai), but with reduced severity in transgenic lines compared to the WT (Fig. 1k). Notably, V5 only produced mild hyperplasic and water-soaked lesions, and petiole abscission, an advanced stage-mark of CC, was never observed (Fig. 1k). Although V4 showed reduced symptom development, the bacterial population was not significantly different from the WT (Fig. 1l). Pathogen growth in the V5 line was consistently reduced in the order of 3 log units (Fig. 1l). In this transgenic line, bacterial spreading and growth were restrained, corroborating symptomatology results.

To assess *EFR*-expressing citrus responses upon *Xfp* infection, a bacterial suspension (10^8 CFU mL^{-1}) of the 9a5c strain (Simpson *et al.*, 2000) was petiole-inoculated on the first leaf of ten transgenic and WT plants. Disease severity and bacterial population assessed by qPCR were evaluated 18 months after inoculation at 5 and 30 cm above the inoculation point (aip). Seventy-one percent of the plants showed

colonization 5 cm aip, but not in more distal parts (Fig. 1m). By contrast, WT and only two transgenic clones colonized distal parts at 30 cm aip (Fig. 1m). Nevertheless, the symptom severity was much lower in transgenic plants compared to WT (Fig. 1m). Transgenic lines without long-distance colonization were symptom-free during the extent of the evaluated time course (Fig. 1m-n). These results indicate that the presence of *EFR* affects bacterial colonization and prevents systemic spread, a process associated with the release of *X. fastidiosa* OMVs (Ionescu *et al.*, 2014). For more information on the methods see https://doi.org/10.5281/zenodo.4723427.

In summary, our results show that the expression of *EFR* in sweet orange confers ligand-dependent activation of defense responses, improving resistance against two citrus bacterial pathogens. With the aim to decrease or avoid the use of agrochemicals, genetic increase of crop resistance is economically viable and sustainable. It opens possibilities and encourages the use of pattern recognition receptors (PRRs) like EFR to confer broad-spectrum resistance as a strategic approach that may support biotechnology citrus breeding programs. This is the first report of the successful transfer of EFR into a perennial crop, increasing resistance to *X. fastidiosa*, a devastating pathogen in citrus and olive groves across Europe and the Americas, for which no genetic or chemical methods are available. Our work describes a genetic strategy to improve *Citrus* resistance and potentially other perennials.

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Conflicts of interest statement

The authors declare no conflict of interest.

Author contribution statement

AAS, SR and CZ designed this research. LKM, NSTS, DMM, RRSN, and KR conducted experiments and analyzed data. LKM and NSTS drafted the manuscript. LKM, NSTS, KR, SR, AAS, and CZ contributed with intellectual input. AAS, SR and CZ provided analytical tools and revised the manuscript. All authors read and approved the final manuscript.

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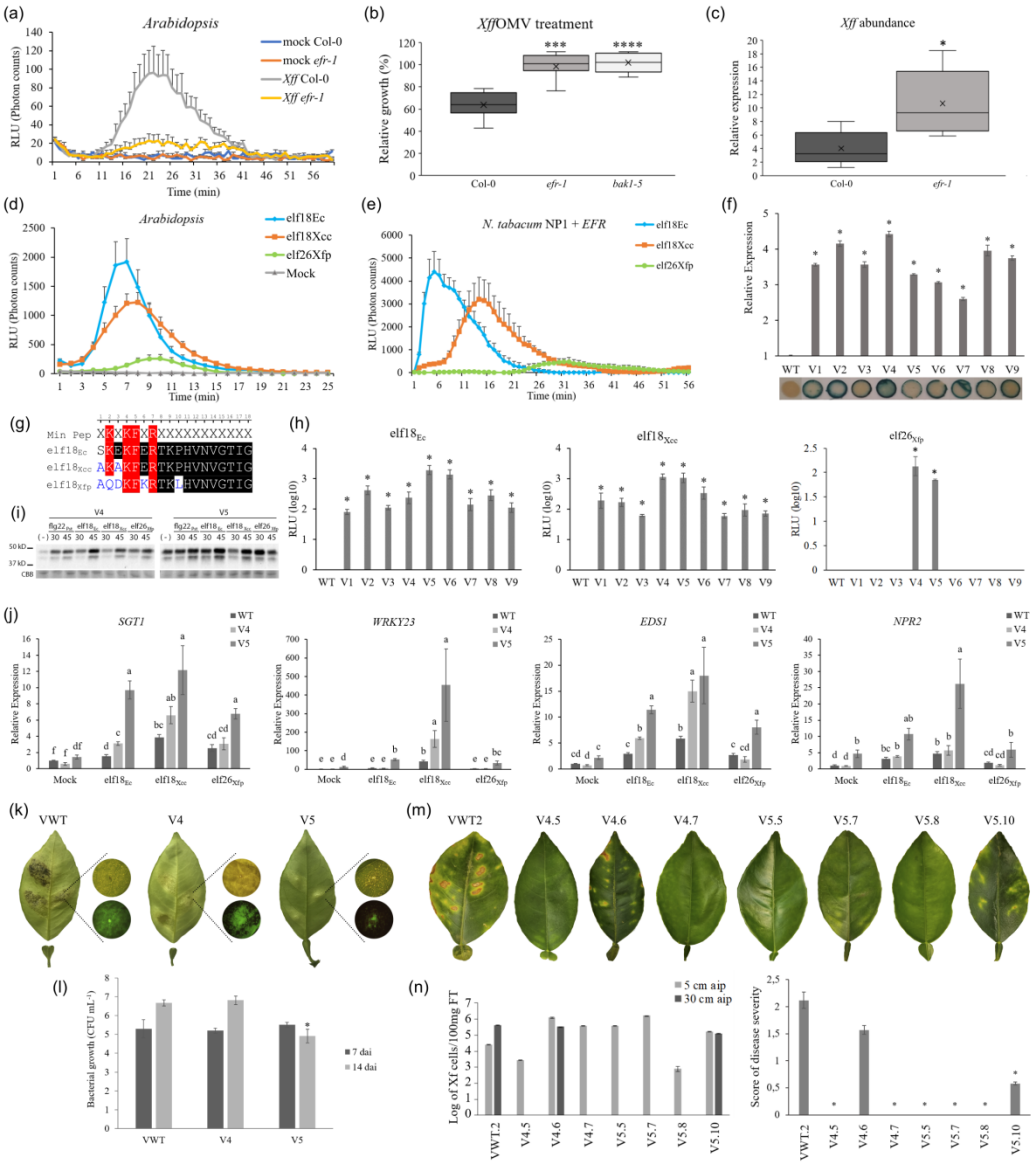
Figure Legends

Figure 1 *Arabidopsis* EFR responds to *Xcc* and *Xfp*, inducing broad-spectrum resistance in transgenic sweet orange. **(a)** *A. thaliana efr-1* is strongly impaired in *Xff*-induced ROS burst (n=10). **(b)** *Xff* OMVs repress seedling growth in *Arabidopsis* wild-type Col-0 but not *efr-1* and *bak1-5.* **(c)** *Xff* bacterial titers are higher in *efr-1* measured as relative expression of the *Xff HL5/HL6* locus 14 days after petiole infection. **(d,e)** ROS production in *Arabidopsis* Col-0 **(d)** and *N. tabacum* expressing *EFR* **(e)** triggered by elf peptides ($elf18_{Ec}$, $elf18_{Xcc}$ and $elf26_{Xfp}$). **(f)** Transgenic integration confirmed by histochemical *gus* assay and relative expression of *EFR* measured by RT-qPCR normalized by the expression of *cyclophilin* in Valencia transgenic citrus lines. **(g)** Alignment of the EF-Tu-derived elf18 sequences from *E. coli*, *X citri* and *X. fastidiosa* compared to the minimal peptide (in red, where X is any amino acid) required for full EFR activation. Amino acids in blue represent substitutions. **(h)** ROS production of transgenic citrus lines in response to elf peptides ($elf18_{Ec}$, $elf18_{Xcc}$ and $elf26_{Xfp}$). **(i)** MAPK activation and **(j)** defense gene induction in citrus-*EFR* lines after elf treatment. **(k)** CC symptom development and **(l)** bacterial growth in detached leaves of citrus-*EFR* lines infected with *Xcc*. Circles represent details in bright-field (upper circle) and under GFP-fluorescence (lower circle). **(m)** CVC symptomatology and **(n)** bacterial population disease severity score 18 months after *Xfp* inoculation. RLU: relative light units. All the experiments were performed three times with at least n=3 with similar results.



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