# Arabidopsis EF-Tu receptor enhances bacterial disease resistance in transgenic wheat

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# Summary

* Perception of pathogen (or microbe)-associated molecular patterns (PAMPs/MAMPs) by pattern recognition receptors (PRRs) is a key component of plant innate immunity. The Arabidopsis PRR EF-Tu receptor (EFR) recognises the bacterial PAMP elongation factor Tu (EF-Tu) and its derived peptide elf18. Previous work revealed that transgenic expression of *AtEFR* in *Solanaceae* confers elf18 responsiveness and broad-spectrum bacterial disease resistance.
* In this study, we developed a set of bioassays to study the activation of PAMP-triggered immunity (PTI) in wheat. We generated transgenic wheat plants expressing *AtEFR* driven by the constitutive rice actin promoter and tested their response to elf18.
* We show that transgenic expression of *AtEFR* in wheat confers recognition of elf18, as measured by the induction of immune marker genes and callose deposition. When challenged with the cereal bacterial pathogen *Pseudomonas syringae* pv. *oryzae*, transgenic EFR wheat lines had reduced lesion size and bacterial multiplication.
* These results demonstrate that *AtEFR* can be transferred successfully from dicot to monocot species, further revealing that immune signalling pathways are conserved across these distant phyla. As novel PRRs are identified, their transfer between plant families represents a useful strategy for enhancing resistance to pathogens in crops.

# Keywords

PAMP/MAMP-triggered immunity; Durable disease resistance; Bacterial halo blight;

Transgenic wheat; Pathogen recognition; Dicotyledon-to-monocotyledon gene-transfer; Immune receptor signaling.

# Introduction

The first line of active defence in the plant immune system involves the recognition of pathogen (or microbe)-associated molecular patterns, PAMPs (or MAMPs)by transmembrane pattern recognition receptors (PRRs) (Boller & Felix, 2009; Schwessinger & Ronald, 2012; Zipfel, 2014). Following detection by PRRs, a series of defence reactions are initiated leading to PAMP-triggered immunity (PTI), which is thought to be sufficient to repel most microbes. Successful pathogens have to evade or suppress PTI, and commonly do so by employing effector proteins. These effectors can in turn be recognised by plant resistance (R) proteins resulting in a strong defence reaction described as effector-triggered immunity (ETI) (Dodds & Rathjen, 2010). *R*-gene mediated resistance has been widely used in breeding wheat and other crops. While many *R*-genes provide near-complete resistance to specific pathogen races, they can easily become ineffective as pathogens mutate or lose the single effector they recognise. Most of the *R*-genes bred into wheat have become ineffective due to appearance of new pathogen races (Keller *et al.*, 2000; Boyd *et al.*, 2013; Dangl *et al.*, 2013). The risk of *R*-genes breaking down has encouraged breeders to search for more durable forms of resistance.

PAMPs are important molecules conserved across microbial taxa that cannot easily be deleted or mutated. Resistance based on PTI is therefore potentially durable and broad-spectrum (Roux *et al.*, 2014). Several PAMP-PRR pairs have been described, and are increasingly being investigated in crop species (Schwessinger & Ronald, 2012; Kawano & Shimamoto, 2013; Wu & Zhou, 2013). For example, chitin is a major constituent of fungal cell walls that is commonly targeted by plant defence systems (Hamel & Beaudoin, 2010). In Arabidopsis (*Arabidopsis thaliana*, At) the LysM domain receptor kinase (RK) CHITIN ELICITOR RECEPTOR-LIKE KINASE 1 (CERK1) is the PRR for chitin (Miya *et al.*, 2007; Wan *et al.*, 2008). In rice (*Oryza sativa*, Os), both the LysM domain-containing receptor-like protein (RLP) CHITIN ELICITOR BINDING PROTEIN (CEBiP) and CERK1 are required for chitin recognition and to mediate resistance to fungal pathogens (Kaku *et al.*, 2006; Kishimoto *et al.*, 2010; Shimizu *et al.*, 2010; Mentlak *et al.*, 2012; Shinya *et al.*, 2012; Hayafune *et al.*, 2014; Kouzai *et al.*, 2014a; Kouzai *et al.*, 2014b). Interestingly, CEBiP and CERK1 also play an important role in chitin recognition and fungal resistance in wheat (Lee *et al.*, 2014). Furthermore, silencing of the *CEBiP* ortholog in barley increases susceptibility to the fungus *Magnaporthe oryzae* (Tanaka et al., 2010), suggesting that CEBiP may be also involved in chitin perception in this crop species. The best characterised bacterial PAMPs recognised by plants are flg22, an epitope within the most conserved region of bacterial flagellin (Felix *et al.*, 1999) and elf18, an epitope within the bacterial elongation factor Tu (EF-Tu) (Kunze *et al.*, 2004). FLAGELLIN SENSING 2 (FLS2) belongs to the leucine-rich repeat (LRR)-RK family XII and recognises flg22 in Arabidopsis, rice, tomato, soybean and grapevine (Gomez-Gomez & Boller, 2000; Robatzek *et al.*, 2007; Takai *et al.*, 2008; Valdés-López *et al.*, 2011; Trda *et al.*, 2014). Arabidopsis EF-TU RECEPTOR (EFR) also belongs to the LRR-RK family XII, but recognises elf18 and is restricted to Brassicaceae (Zipfel *et al.*, 2006; Boller & Felix, 2009; Lloyd *et al.*, 2014). Recently, it has been reported that rice can recognise bacterial EF-Tu via an epitope other than elf18, but the corresponding PRR is currently unknown (Furukawa *et al.*, 2014). Within monocot plant species, rice Xa21 also belongs to LRR-RK family XII and confers durable resistance to bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae*, via the recognition of a yet unidentified PAMP (Song *et al.*, 1995; Bahar *et al.*, 2014).

Since PTI contributes to basal and non-host resistance and PAMP perception mediates recognition of whole class of microbes, the transfer of PRRs might confer resistance to current pathogens into crops by introducing recognition of previously undetected epitopes. Indeed, expression of individual PRR has been shown to increase resistance to fungal and bacterial pathogens, even across the monocot and dicot classes, such as *AtEFR* in tomato (Lacombe *et al.*, 2010), and *OsXa21* in orange, tomato, banana and Arabidopsis(Mendes *et al.*, 2010; Afroz *et al.*, 2011; Tripathi *et al.*, 2014; Holton *et al.*, 2015). To test whether EFR could confer elf18 responsiveness in wheat, we first established methods to assess PAMP responses in this crop using flg22 and chitin. We then created transgenic wheat plants expressing *AtEFR*, and demonstrated that this confers responsiveness to elf18 and enhanced resistance to *Pseudomonas syringae* pv. *oryzae*, a bacterial pathogen of cereals causing halo blight (Kuwata, 1985; Rudolph & von Kietzell, 1997).

# Materials and Methods

***Chemicals***

Unless specified otherwise, chemicals and oligonucleotides were purchased from Sigma ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)), PCR enzymes from Qiagen (www.qiagen.com), cloning vectors and kits from Invitrogen (www.lifetechnologies.com), media from Lab M (Heywood, Lancashire, UK), and Nystatin from Melford (Chelsworth, UK).

## *Plant materials and growth condition*

All experiments were in the background of spring wheat (*Triticum aestivum* L.) line NB1 (http://www.nickersondirect.co.uk). Line 5\_0A, which has undergone the same manipulations as the *AtEFR* transformed plants, was used as a non-transgenic control. Plants were grown in cereal mix, (Peat 40%, Soil 40% Grit 20% and nutrients) in a glasshouse for seeds and in a growth cabinet (23°C, 16 h light/18°C, 8 h dark) for 3-4 weeks for bioassays.

## *Generation of AtEFR transgenic wheat lines*

Full-length *AtEFR* (AT5g20480; NM\_122055) was amplified from *A. thaliana* genomic DNA by PCR reaction with primers 5’-caccaccATGaagctgtccttttcacttgtttt-3’ and 5’-ctacatagtatgcatgtccgtattt-3’ using Phusion (Finnzymes.com) and cloned into pENTR/D/TOPO. The DNA fragment was transferred using the Gateway Clonase LR reaction into pSc4ActR2R1-SCV (Biogemma.com) under control of the rice actin promoter and the *Agrobacterium tumefaciens* nos terminator (Supporting Information Fig. S1; Methods S1). The *A. tumefaciens* supervirulent strain EHA105 (Hood *et al.*, 1993) was used for transformation of the T-DNA region of pActEFR into wheat line NB1 (Risacher *et al.*, 2009). Rooted plantlets regenerated from callus material selected on 25 mg l-1 Geneticin G418 were transferred to Jiffy-7 peat pellets, then to soil and grown to maturity. A rapid NaOH boiling method was modified for 96-well plates (Fig.S3; Methods S2) from Klimyuk (1993) and used for genotyping to identify homozygous transgenic families, using *AtEFR* internal primers and *TaEF-1* primers as a positive control (Table S1).

## *Quantification of PAMP-induced gene expression*

Strips (25 mm long) were cut from wheat leaves and placed into 2ml tubes (3 per tube) with dH2O and pre-infiltrated by vacuum for 3 x 45 s. Leaf strips were left to recover in the growth cabinet for 16 h to avoid gene expression response to water infiltration. After recovery, water was replaced by fresh water (control) or a PAMP solution, 500 nM flg22, 300 nM elf18 (Peptron.co.kr) or 1 g l-1 chitin (Yaizu Suisankagaku Industry Co.). Samples were drained and flash frozen in liquid N2 at different time points and stored at -20°C.

For total RNA extraction, leaf samples were crushed with 5 mm metal balls in dry ice in a Tissuelyser II (Qiagen) and mixed with 1 ml TRI Reagent by vigorous vortexing. After 10 min at room temperature, 100 µl 1-bromo-3-chloropropane (BCP) was added and each sample was vigorously vortexed for 15 s. Samples were incubated at room temperature for 10 min and centrifuged at 12,000 ***g*** for 10 min at 4°C. RNA from the aqueous layer was precipitated with isopropanol at room temperature for 5 min, and centrifuged at 12,000 ***g*** for 8 min at 4°C. Pellets were washed with 75% ethanol twice, and re-suspended in 60 μl of RNase-free water. After treatment with DNase Turbo DNA-Free (Ambion), RNA was quantified in a spectrophotometer (Nanodrop2000, Thermo Scientific).

For quantitative reverse-transcription PCR (qPCR) analysis, first-strand cDNA was synthesized from total RNA using SuperScript III. One microgram of total RNA primed with oligo(dT)20 and random hexamers was used in a 20 μl reaction, following the supplier's instructions. The resulting cDNA was analyzed using the SYBR Green JumpStart Taq ReadyMix (Sigma) on a Chromo4 real-time PCR system (MJ/Bio-Rad) using 0.5 μl of cDNA per 20 μl PCR. A set of primers for genes responding during PTI was defined (Table S1; Note S1), and used at a final concentration of primers of 0.2 μM and an annealing temperature of 60°C. Wheat elongation factor-1α (*TaEF-1α*) was used as the endogenous control and for normalisation of gene-expression.

## Callose staining and microscopy analysis

Leaves were harvested 24 hours after elf18 infiltration by needle-less syringe, and cleared in EtOH at 50%, 70% and then 100%. The cleared leaves were rehydrated in 50% EtOH and in 67 mM K2HPO4 pH 12 for 1 h. Callose deposition was detected after staining the leaves with a solution of 0.01% (w/v) aniline blue in 30% 67 mM K2HPO4 pH 12 and 70% glycerol for 1 hour with an epifluorescence microscope (Leica DM6000, BP 340 to 380 nm, LP 425 nm). For each treatment, four leaves were examined and on each leaf 20 microscopic fields were counted.

## *Bacterial infections*

*Pseudomonas syringae* pv. *oryzae* strain Por36\_1 (Hwang *et al.*, 2005) was used to generate spontaneous mutants resistant to rifampicin (Note S2, Fig. S2). The derived strain Por35\_1rif was grown for 24 h at 28°C on KB agar (King *et al.*, 1954) containing 50 mg l-1 rifampicin and 25 mg l-1 nystatin (KBArifnys). Bacteria were re-suspended in 5% KB liquid medium to an OD600nm = 0.02. For bacteria-induced gene expression, this bacterial suspension was applied to leaf strips as the PAMP solution described above.

For wheat inoculation, six 1-mm holes were punctured in the 3rd leaf of five plants with a pin and a 2 µl droplet of Por36\_1rif suspension or control treatment with 5% KB was applied on each hole. The plants were sealed in a plastic bag and incubated in a growth cabinet (23°C 16h light/ 18°C 8h dark) for 4 days. Disease symptoms appeared as yellow concentric circles and were assessed by direct measurement (of the lesion size) and by counting bacteria after extraction and serial dilution.

Three sections of inoculated leaf, each of which contained a complete lesion, were disrupted with two metal balls (5 mm) in 500 µl KB in a GenoGrinder (2x20 s, 1250 spm) to release bacteria from the leaf apoplast. A 10-1-to-10-6 dilution series was made in KB and 10 µl from each dilution was spotted on KBArifnys and incubated at 28°C for 20 h. The number of colonies was counted to calculate the number of colony forming units (cfu) per inoculation site. Four independent experiments were performed.

# Results and discussion

## Flg22 and chitin induce PTI marker genes in wheat

For our aim of developing tools to study PTI in wheat, we tried to measure the ROS burst in response to PAMPs but never obtained reproducible results using established methods (Note S3). Therefore, we sought to select marker genes, based on homology to known marker genes in other plant species, or on the involvement of these genes in wheat defence responses (Table S1; Fig. 1a). These include wheat homologues of the PRRs CEBiP (KJ866877.1) and FLS2, which are expressed but barely upregulated by PAMPs, the syntaxin *ROR2*, a homologue of *PEN1* in Arabidopsis and *HvRor2* in barley, which is involved in defence against pathogens (Collins *et al.*, 2003), as well as *TaMPK3*, which encodes a MAP kinase involved in resistance to *Mycosphaerella graminicola* (Rudd *et al.*, 2008). *PDR2* (Ta.21281.1.s1) is induced by adapted and non-adapted *Magnaporthe* isolates on wheat (Tufan *et al.*, 2009). A homologue of the transcription factor *WRKY23* is induced by elf18 in Arabidopsis (Kunze *et al.*, 2004). The ubiquitin ligase genes *PUB23*-like and *CMPG1-like* were highly induced, as were their respective homologues *PUB23* in Arabidopsis (Trujillo *et al.*, 2008) and the immediate-early fungal elicitor responsive gene *CMPG1* in parsley (Kirsch *et al.*, 2001). The wheat homologues of cupredoxin and CamBP are particularly good markers genes due to their robust up-regulation upon PAMP treatment. Treatment of non-transgenic control wheat (5\_0A) with flg22 and chitin increased levels of transcripts for most genes tested (Fig. 1a), revealing that selected genes can be used as PTI marker genes in wheat.

## Selection of transgenic wheat lines expressing *AtEFR*

Primary transformed wheat plants were selected in tissue culture by their ability to regenerate in the presence of G418. Two T1 plants from each of 3 independent transformation events were selected for presence of *AtEFR* by PCR. T2 seedlings of these plants were used to verify the presence (Fig. 2a) and expression (Fig. 2b; Fig. S3) of the *AtEFR* transgene. T3 seedlings were used in segregant analysis. Nine out of twenty four T2 plants tested had 100% transgenic offspring, which is consistent with the expected result of 1 out of 3 homozygous lines for confirmed transgenic parents. Six independent homozygous *AtEFR* transformants were generated with varying levels of expression (Fig. S3) and four of these (4\_2E, 5\_4C, 6\_1D and 6\_2C) were selected for further study (Fig. 2). Importantly, induction of the selected marker genes (Fig. 1a) by flg22 and chitin still occurred in transformant 5\_4C (Fig. 1b), which expressed the highest level of *AtEFR* transcript (Fig. 2b), indicating that ectopic EFR expression does not interfere with FLS2 and CEBiP functions. Notably, *AtEFR* expression in 5\_4C was only 16±11% that of the endogenous*TaFLS2-like* gene (Figs. 1 and 2).

## Transgenic expression of *AtEFR* in wheat confers elf18 responsiveness

We compared PAMP-induced gene expression and callose deposition in the non-transgenic control wheat line 5\_0A and *AtEFR* transgenics following elf18 infiltration (Fig. 3 and Fig. S4). Significant increases for all marker genes, except for *ror2*, were measured in 5\_4C compared to 5\_0A at 30 or 180 min after elf18 treatment (Fig. 3a,b). Notably, the gene-induction in response to elf18 in 5\_4C was weaker than that to flg22 and chitin (Fig. 3a,b and Fig. 1a,b), consistent with the lower level of expression relative to *TaFLS2*-like. Callose deposits were observed after elf18 infiltration, with a significant increase in 5\_4C compared to 5\_0A (Fig. 3c,d). These results demonstrate that elf18 induced PTI responses specifically in 5\_4C, indicating that *AtEFR* is functional in transgenic wheat.

Since flg22 can induce PTI responses in wheat (Fig. 1a), FLS2, EFR and Xa21 have homologues in wheat (Tan *et al.*, 2011) (Fig. S5), and EFR and FLS2 share many signalling components in Arabidopsis (Macho & Zipfel, 2014), we assume that components required for AtEFR function are also present in wheat. These might also contribute to the PAMP response after exposure to whole bacteria (Fig. 3). A key signalling component acting downstream of AtFLS2 and AtEFR is the co-receptor SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 /BRASSINOSTEROID-ASSOCIATED KINASE1 (SERK3/BAK1) (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Roux *et al.*, 2011). Notably, cereals seem to have no clear BAK1/SERK3 orthologs, but the SERK protein OsSERK2 has been shown to be required for Xa21 function in rice (Chen *et al.*, 2014). Interestingly, genes with high homology to OsSERK2 are present in the wheat genome (Fig. S6), (Singla *et al.*, 2008; Cantu *et al.*, 2013), presumably supporting signalling through TaFLS2 and AtEFR.

## Transgenic expression of AtEFR in wheat confers increased resistance to *Pseudomonas syringae* pv. *oryzae*

To test if *AtEFR* expression confers bacterial recognition in wheat and subsequent anti-bacterial immunity, we challenged non-transgenic (5\_0A) and transgenic (5\_4C) wheat lines with the cereal pathogen *Pseudomonas syringae* pv. *oryzae* strainPor36\_1rif. Notably, the elf18 amino acid sequence of *P. syringae* pv. *oryzae* (AKEKFDRSLPHVNVGTIG) is recognised by AtEFR in Arabidopsis (Lacombe *et al.*, 2010), so AtEFR expressed in wheat is expected to recognise Por36\_1rif-derived EF-Tu and initiate PTI responses. PTI was first evaluated by gene induction following inoculation with Por36\_1rif (Fig. 4a,b). The marker genes were induced both in 5\_0A and 5\_4C (Fig. 4a,b), indicating the detection of bacterial PAMPs other than EF-Tu, which could include flagellin or other molecules. However, gene induction was stronger in 5\_4C (Fig. 4a,b), consistent with an additional response to EF-Tu conferred by *AtEFR*.

Lastly, we tested if the gain of elf18 responses in *AtEFR*-expressing plants culminates in increased resistance to Por36\_1rif. All transgenic wheat lines and the control line (5\_0A) were infected by Por36\_1rif. Interestingly, the *AtEFR* lines showed different disease development with smaller, paler lesions when compared to 5\_0A (Fig. 4c). We attribute the altered lesion colour in the *AtEFR* transgenics to a stronger manifestation of innate defence responses. Three of the four selected *AtEFR* transgenic lines were significantly more resistant to Por36\_1rif than 5\_0A. The greatest reduction in lesion size (Fig. 4d) and bacterial density (as measured by CFU; Fig. 4e) was in line 5\_4C, consistent with the highest expression level of *AtEFR* (Fig. 2b). Lesion size and bacterial density in 6\_2C was not significantly lower than the control line (Fig. 4c-e), consistent with low *AtEFR* expression (Fig. 2b). Transgenic 4\_2E and 6\_1D had intermediate lesion size, bacterial density (Fig. 4c-e) and *AtEFR* expression (Fig. 2b). Together, these data demonstrates that *AtEFR* expression levels in transgenic wheat correlate with increased resistance to the pathogenic bacterium *P. syringae* pv. *oryzae*.

# Conclusion

Our results show that the PRR EFR from the dicot Arabidopsis can be successfully transferred to wheat, a monocot plant species. Stable transgenic expression of *AtEFR* led to elf18 responsiveness and increased resistance to the bacterial pathogen *P. syringae* pv. *oryzae*. These results echo those previously obtained when *AtEFR* was expressed in the *Solanaceae* plants *N. benthamiana* and tomato (Lacombe *et al.*, 2010), as well as recent results published during the revision of this manuscript showing that expression of EFR in the monocot plant rice confers elf18 responsiveness and increased resistance to bacteria (Lu *et al.*, 2014; Schwessinger *et al.*, 2014).

The gain in elf18 responsiveness indicates that there are sufficient shared signalling components between monocots and dicots for AtEFR to integrate into the wheat PTI pathway. As more PAMP/PRR pairs are identified, transferring PTI responses between dicot and monocot species increases the opportunities for developing durable, broad-spectrum resistance to plant diseases in crops.

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# Supporting information

The following Supporting Information is available for this article:

**Fig. S1** Construct for over-expression of *AtEFR* in wheat.

**Fig. S2** Growth curve of *Pseudomonas syringae* pv*. oryzae* Por36\_1rif on wheat and barley.

**Fig. S3** Genotyping and *AtEFR* expression in additional transgenic T3 wheat lines.

**Fig. S4** *AtEFR* expression in wheat confers elf18 responsiveness in multiple transgenic wheat lines.

**Fig. S5** Phylogenetic analysis of the kinase domain of wheat proteins with homology to known receptor kinases.

**Fig. S6** Phylogenetic analysis of the kinase domain of wheat proteins with homology to BAK1 and other SERKs.

**Table S1** Genes and primers used in the quantitative real-time PCR (qPCR) studies and genotyping.

**Methods S1** Vectors and strains used for generation of AtEFR transgenic wheat lines.

**Methods S2** Extraction of genomic DNA from wheat for genotyping by PCR using alkaline hydrolysis.

**Notes S1** Cloning of *CEBiP* cDNA from wheat.

**Notes S2** Development of bioassays on wheat with rifampicin resistant *Pseudomonas syringae* pv. *oryzae* strain Por36\_1.

**Notes S3** Bioassays to determine reactive oxygen species (ROS) burst in response to PAMPS in wheat.

**Supporting Information References**

# Figure legends

## Figure 1. Identification of PAMP-responsive marker genes in wheat.

Gene expression induced by 500 nM flg22 and 1 g l-1 chitin in (**a**) 5\_0A (control) and (**b**) *AtEFR* transgenic line 5\_4C after 60 min determined by quantitative RT-PCR and presented as fold induction relative to water treatment. Results are mean values ± SEM from three replicate experiments.

## Figure 2. Genetic characterization of selected transgenic *AtEFR* T3 wheat lines.

(**a**) PCR analysis of 5\_0A (control) and transgenic lines indicates the presence (+) or absence (-) of the introduced *AtEFR* gene and wheat genomic DNA (*TaEF-1α* was used as positive control for DNA presence). (**b**) Basal *AtEFR* transcription level in transgenic lines was measured by quantitative RT-PCR. Data presented are normalised against the wheat housekeeping gene *TaEF-1α.* Results are mean values ± SD from three replicate experiments.

## Figure 3. *AtEFR* expression in wheat confers elf18 responsiveness.

Gene expression induced at (**a**) 30 min, and (**b**) 3 h after induction with 300 nM elf18 in 5\_4C and 5\_0A was determined by quantitative RT-PCR. Expression is presented as fold induction relative to water treatment. Results are mean values ± SD from three replicate experiments \* Significant difference between 5\_4C and 5\_0A using Student’s t-test (*p* < 0.05).

(**c**) Callose accumulation in 5\_4C 24 h after infiltration with 300 nM elf18. Arrowheads show deposition of callose. Bar indicates 100 m. (**d**) Number of callose depositions, quantified by counting 20 microscopic fields (400x magnification) per leaf strip. Results are mean value ± SD from four replicate experiments. Different letters above bars indicate significant differences in the number of callose depositions at p < 0.05 (one way ANOVA, Tukey post hoc test).

## Figure 4. *AtEFR* transgenic wheat lines show elevated resistance to *Pseudomonas syringae* pv. *oryzae*.

*Pseudomonas syringae* pv. *oryzae* Por36\_1rif-induced gene expression at (**a**) 30 min, and (**b**) 3 h in 5\_4C and 5\_0A was quantified by quantitative RT-PCR. Induction is presented as fold induction relative to water treatment. Results are mean values ± SD from three replicates \* Significant difference between 5\_4C and 5\_0A using Student’s t-test (*p* < 0.05). (**c**) Disease symptoms photographed at 4 days post-inoculation (dpi). Plants were inoculated with Por36\_1rif (concentration OD600nm 0.02 in 5% KB) by pipetting 2 µl bacterial suspension on the pinpricks and incubated at high humidity for 4 days. Mock indicates the control treatment: pinpricks inoculated with 5% KB. (**d**) Bacterial lesion measured at 4 dpi. Results are mean values ± SEM (n = 25 based on 5 infected sites per leaf from 5 plants). \* Significant difference from 5\_0A using Student’s t-test (*p* < 0.05). (**e**) Quantification of Por36\_1rif at 4 dpi. Results show colony-forming units (CFU) means ± SEM (n = 20 based on 4 lesions per leaf from 5 plants); the multiplier (10-7) is that by which the original number

has to be multiplied to yield the number given in the figure. \* Significant difference from 5\_0A using Student’s t-test (*p* < 0.05). This experiment was repeated three times with similar results.

## *New Phytologist* Supporting Information

Article title: Arabidopsis EF-Tu receptor enhances bacterial disease resistance in transgenic wheat

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**Supporting Information References**

**Fig. S1** Construct for over-expression of *AtEFR* in wheat The *AtEFR* in sequence was recombined with the binary destination vector pSc4ActR2R1-SCV (Biogemma) in a Gateway Clonase LR recombination reaction (Invitrogen) to create pActEFR. This Gateway compatible binary plasmid is a super clean vector (Firek *et al.*, 1993), containing the rice *Act1* promoter and 5’ intron, and the *nos* terminator for constitutive expression of a gene sequence recombined into the gateway aatR2-aatR1 sites, plus the *NPTII* (*neomycin phosphotransferase II*) gene under the control of the *subterranean clover stunt virus* Sc4 promoter (Schunmann *et al.*, 2003) and *Arabidopsis thaliana* *FAD2* intron (Okuley *et al.*, 1994; Lelong *et al.*, 2004) for selection on Geneticin G418 in tissue culture. The resulting plasmid pActEFR was introduced by electroporation into *Agrobacterium tumefaciens* supervirulent strain EHA105 (Hood *et al.*, 1993).

**Fig. S2** Growth curve of *Pseudomonas syringae* pv. *oryzae* Por36\_1rif on wheat and barley. The third leaf of (**a**) wheat 5\_0A or (**b**) barley (Golden Promise) was inoculated with Por36\_1rif (concentration OD600nm 0.02 in 5% KB) by pipetting 2 µl bacterial suspension on the pinpricks and incubated at high humidity. Results show colony-forming units (CFU) means ± SEM (n = 20 based on 5 lesions per leaf from 4 plants). Mock indicates leaves subjected to pinprick but not inoculated with bacteria, sampled after handling all other leaves; inoculum indicates the initial inoculum (2 µl bacterial suspension) pipetted directly in the well off the 96-well plate used for extraction of the leaves; 0dpi is the amount of bacteria recovered from the leaves within 30 min after inoculation. Data presented are based on 2 repeat experiments. (**c**) Correlation between lesion size and bacterial counts (log10). The size of individual lesions from day1,2,3 in panel (b) was measured and plotted against the corresponding bacterial counts, showing a positive correlation between size and presence of bacteria.

**Fig. S3** Genotyping and *AtEFR* expression in 5\_0A and additional transgenic T3 wheat lines. All pairs with the same numbers are from the same transformation, letters indicate different segregating families from a transformation event. Genomic DNA was extracted according to Methods S2 and 1 µl of the lysate was used for genotyping with *AtEFR* internal primers and *TaEF1α* primers as a positive control. (**a**) PCR analysis of transgenic lines indicates the presence (+) or absence (-) of the introduced *AtEFR* gene and wheat genomic DNA (*TaEF1α* was used as positive control for DNA presence). (**b**) *AtEFR* transcription level of transgenic lines was measured by quantitative RT-PCR. Data present in normalisation normalised by wheat endogenous gene *TaEF-1α*. Both families from 4\_1, 5\_4 and 6\_2 show comparable expression level (medium, high and low respectively).

**Fig. S4** *AtEFR* expression in wheat confers elf18 responsiveness in multiple transgenic wheat lines. Gene expression in transgenic wheat lines at 30 min after infiltration with water (white bars) or 300 nM elf18 (grey bars) was determined by quantitative RT-PCR. Induction is presented as expression levels relative to *TaEF1α*. Results are mean values ± SD from three replicate samples. Induction in the high *AtEFR-*expressing line 5\_4C is higher than in 4\_2E and 6\_1D, which have intermediate expression of *AtEFR* and induction is nearly absent in the transformant 6\_2C, which shows lowest expression levels of *AtEFR*.

**Fig. S5** Phylogenetic analysis of the kinase domain of wheat proteins with homology to known receptor kinases (RKs). Wheat and barley genes annotated as homologues of Arabidopsis *AtCERK1,* *AtEFR*, *AtFLS2* or rice *OsFLS2*, *OsXa21* and *OsCERK1* were retrieved from public databases using text searches. Wheat genes with homology to *AtEFR, AtFLS2*, *OsFLS2*, *OsXa21* and *OsCERK1* were obtained from genbank at NCBI (<http://www.ncbi.nlm.nih.gov>) and TriFLDB (<http://trifldb.psc.riken.jp/v3/index.pl>; Mochida *et al.*, 2009) by BLAST searches. A non-redundant list of obtained protein sequences was trimmed to contain only the kinase domain and aligned using MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred by using the Maximum Likelihood method basedon the Poisson correction model in MEGA5. Gene-identifiers for RKs from wheat, barley, rice and Arabidopsis are preceded by a two-letter species denominator: Ta, *Triticum aestivum*; Hv, *Hordeum vulgare*; Os, *Oryza sativa;* and At, *Arabidopsis thaliana*. Presented are all RKs clustering with the Arabidopsis LRR-RK family XII (Shiu & Bleecker, 2001) and the CERK1 homologues. Proteins with a published role in PTI are indicated with arrows. The branch of cereal RKs closest to *AtEFR* is the one containing *OsXa21*, a functional PRR with homologues in wheat and barley (Cantu *et al.*, 2013).

**Fig. S6** Phylogenetic analysis of the kinase domain of wheat proteins with homology to BAK1 and other SERKs Wheat and barley genes annotated as a member of the Somatic Embryogenesis Receptor Kinase (SERK) family or as homologues of *AtSERK3* or *AtBAK1* were retrieved from public databases using text searches. Wheat genes with homology to *AtSERK1-5* or *OsBAK1* (Os08g0174700; (Li *et al.*, 2009)) were obtained from Genbank at NCBI (<http://www.ncbi.nlm.nih.gov>) and TriFLDB (<http://trifldb.psc.riken.jp/v3/index.pl>; (Mochida *et al.*, 2009)) by BLAST searches. A non-redundant list of obtained protein sequences was trimmed to contain only the kinase domain and aligned using MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 1.14575598 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Gene-identifiers for genes from wheat, barley, rice and Arabidopsis are preceded by a two-letter species denominator: Ta, *Triticum aestivum*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*, and At, *Arabidopsis thaliana*. No direct homologue of *AtBAK1* (arrow) could be identified in wheat but there are several candidate proteins with high homology to the proposed rice candidates (\*). The accessions gi|188474275 and gi|124303893 were annotated as TaBAK1 and HvBAK1 by their original depositors to the databases. They probably were the closest known homologs of AtBAK1/AtSERK3 at the time, however, the increase in available sequence data has allowed them to be classified as homologs of OsSERK2, which is required as a co-receptor for Xa21 (Chen *et al.*, 2014) and is closer related to AtSERK2.

**Table S1** Description of genes studied and primers used in the quantitative real-time PCR (qPCR) studies and genotyping (g)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Description** | **Accession** | **source** | **Ta locus name 4)** | **Forward primer** | **Reverse primer** |
| *AtEFR*g | *Arabidopsis thaliana* EF-Tu Receptor | AY075690 | 1) |  | CCAGTGATGGTAACCCATCTG | TAGCTGCAGCCACATATCCA |
| *AtEFR* | *Arabidopsis thaliana* EF-Tu Receptor | AY075690 | 1) |  | GGAGGCCAACCATCAATACA | GCTCGTGCAACCCGATAATA |
| *EF-1α* | Elongation factor 1-alpha | M90077 | 1) |  | ATGATTCCCACCAAGCCCAT | ACACCAACAGCCACAGTTTGC |
| *FLS2* | homologous to Arabidopsis and rice Flagellin Sensing2 |  |  | Traes\_2BL\_6E8764762 | CGACGTCTCTGACGAATCTTG | GTTGTGGATGACGAGCTTCTG |
| *CEBiP* | homologous to rice and barley Chitin Elicitor Binding Protein | KJ866877.1 | 1) |  | GTCTTCCACCTCGCCTACATC | AACCTGGTCCTTCTGCAGTGT |
| *MPK3* | MAP Kinase 3 | AF079318.1 | 1) |  | ACCCTTACCTAGAGCGGCTTC | ACTCCAGGGCTTCGTTGAATA |
| *Ror2* | syntaxin, homologous to barley Ror2 and Arabidopsis PEN1 | tplb0005g03 | 2) |  | TCGTGCTCAAGAACACCAAC | AATCGAGTGGCTCAACGAAC |
| *UGE2* | homologous to barley and rice UDP-glucose 4-epimerase |  |  | Traes\_5DL\_FF28DAF89.1 | TCCATATGGCAGAACCAAGC | CGTGGGTCTTCACCAAGGTA |
| *WRKY23-like* | WRKY-transcription factor |  |  | Traes\_1DL\_46428511F.1 | GAGCGTAGACGTCAGCACCA | CACGGATGCTAATGGCCACC |
| *PDR2* | Unigene Ta.21281.1.S1\_at Pleiotropic Drug Transporter2, homologous to PDR1 | Ta.21281.1.S1\_at in Tufan (2009) | 3) | Traes\_5DL\_F292F9EA4.1 | GTGCAGGGGATTCAGTACACA | GTATGTTTGCGAGCATGGAAG |
| *PUB23-like* | Homologous to barley and arabidopsis E3 ubiquitin-protein ligase PUB23 | BQ743320.1 | 1) | [Traes\_3B\_0B86BCF93](http://plants.ensembl.org/Triticum_aestivum/Gene/Summary?g=Traes_3B_0B86BCF93) | CGTTCATCAGAATGCTCAGCTG | TTCTCTTTTGTAGGCACGAACCA |
| *CMPG1-like* | Homologous to arabidopsis E3 ubiquitin-protein ligases and parsley EFE (Immediate-early fungal elicitor protein CMPG1) |  |  | [Traes\_4AS\_068ACAA031](http://plants.ensembl.org/Triticum_aestivum/Gene/Summary?g=Traes_4DL_5ABC60D83) | GGACGCAACCAAGGAGAAGA | TTGAGCCCTCTGAAGTCCAT |
| *Cupredoxin* | homologous to cupredoxins or blue copper proteins (BCP) | FJ459810.1 | 1) |  | AGCGGTAACCTACAACGTCG | GACGATGTCATCACCCACGT |
| *CamBP-like* | Contains motives of Calmodulin Binding Protein |  |  | [Traes\_5BS\_4DE52F9BA](http://plants.ensembl.org/Triticum_aestivum/Gene/Summary?db=core;g=Traes_5BS_4DE52F9BA;r=IWGSC_CSS_5BS_scaff_2269518:489-3288;t=Traes_5BS_4DE52F9BA.2) | CGCGTTCGAGGAGAAACAAG | CGTACCTTGACCAGCCTTGT |
|  |  |  |  |  |  |  |
| 1) http://www.ncbi.nlm.nih.gov/genbank/ | |  |  |  |  |  |
| 2) http://trifldb.psc.riken.jp/v3/index.pl | |  |  |  |  |  |
| 3) http://www.plexdb.org/index.php | |  |  |  |  |  |
| 4) http://plants.ensembl.org/Triticum\_aestivum/Info/Index | | |  |  |  |  |

**Methods S1 Vectors and strains used for generation of AtEFR transgenic wheat lines.**

To create pActEFR (Figure S1), the cloned full-length genomic DNA fragment of AtEFR in pENTER/D/TOPO was recombined with the binary destination vector pSc4ActR2R1-SCV (Biogemma) in a Gateway Clonase LR recombination reaction (Invitrogen). This Gateway-compatible binary plasmid is a super clean vector (Firek *et al.*, 1993), containing the rice Act1 promoter and 5’ intron, and the nos terminator for constitutive expression of a gene sequence recombined into the gateway aatR2-aatR1 sites, plus the NPTII (neomycin phosphotransferase II) gene under the control of the subterranean clover stunt virus Sc4 promoter (Schunmann *et al.,* 2003) and *Arabidopsis thaliana* FAD2 intron (Okuley *et al.*, 1994; Lelong *et al.*, 2004) for selection on Geneticin G418 in tissue culture. The resulting plasmid pActEFR was introduced by electroporation into the *Agrobacterium tumefaciens* supervirulent strain EHA105 (Hood *et al.*, 1993).

**Methods S2 Extraction of genomic DNA from wheat for genotyping by PCR using alkaline hydrolysis.**

A rapid NaOH boiling method was modified for 96-well plates from Klimyuk (1993). The second leaf of seedlings from each line was sampled (2x2 mm) to isolate DNA in a 96-well plate. Leaf tissue was kept at -20 °C overnight, then 30 µl 250 mM NaOH was added to each well and the plate was heated at 96°C for 10 min in a PCR machine. The sample was neutralised with 30 µl 250mM HCl and 30 µl 0.5 M Tris‑HCl (pH 8, 0.25% v/v Triton 100). After heating at 96°C for 2 min, 1 µl of the lysate was used for genotyping with *AtEFR* internal primers and *TaEF1α* primers as a positive control (Fig. S3).

**Notes S1** **Cloning of *CEBiP* cDNA from wheat**

To find sequences in wheat with homology to CEBiP we used the Chinese Spring bread wheat genome survey sequencing reads that are publicly accessible as part of a collaboration between the John Innes Centre and the Universities of Bristol and Liverpool at: <http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/search_reads.php>. We blasted the *HvCEBiP* sequence (AK359591.1) to the assembled reads and used the obtained sequences to design primers for nested amplification of *TaCEBiP* from cDNA from the wheat variety Renan (Tufan *et al.*, 2009). We used the following primers in the UTRs for the first PCR using Phusion high-fidelity DNA polymerase (Finnzymes.com) at an annealing temperature of 61°C: AACGCCGCAGTCAAACGC and CGAAAAGCTCGACAAACCGG. The resulting PCR products were diluted 50 times in water and used for the second, nested, PCR using Coraltaq with the following primers: TCGCCtACATCGTCGACGGC and TCAAAGGAAGCATACCAAGAT in a touchdown protocol with 4 cycles at an annealing temperature of 66 °C preceding 35 cycles at an annealing temperature of 61 °C, extension was always at 72 °C for 1 min. The obtained fragment was gel-purified using the QIAEX II Gel Extraction Kit (Qiagen) and ligated into pCR8/TOPO and transformed by heatshock into TOP10 cells according to suppliers instructions. The obtained fragment was sequenced and contained a fragment of *TaCEBiP* which was submitted to the database with accession number KJ866877.1 This cDNA fragment of *TaCEBiP* has 93% and 76% homology with *HvCEBiP* and *OsCEBiP* respectively. The corresponding protein sequence has 86% and 65% identity with HvCEBiP and OsCEBiP respectively. The cDNA has 99.8% homology to AK331304.1, which has since been identified as *CEBiP* in wheat by Lee *et al.* (2014) and encodes an identical protein. The KJ866877.1 cDNA sequence was used to generate qPCR primers (Table S1).

**Notes S2** **Development of bioassays on wheat with rifampicin resistant *Pseudomonas syringae* pv. *oryzae* strain Por36\_1**

We developed bioassays for the pathosystem *Pseudomonas syringae* pv. *oryzae* strain Por36\_1 (Kuwata, 1985) on wheat with an easy and reliable protocol for inoculation and quantification of resulting bacterial growth. Since Por36\_1 has originally been isolated from rice and was reported to infect rice, barley, oat and kidney bean but cause symptoms without *in planta* growth on wheat variety Kitakamikomugi (Kuwata, 1985) we adapted inoculation methods and tested them on wheat and barley. By wounding with a pinprick and pipetting a 2-µL droplet of inoculum (OD600=0.02 in 5% KB) we obtained inoculation sites with a fixed position and fixed amount of inoculum per site. We then extracted bacteria from leaf strips containing the whole area around the lesion site, thereby recovering all the bacteria grown from the initial inoculum. Using this method we were able to obtain symptoms and recover bacteria from 10 different wheat varieties (Bryant, 2013). Symptoms on the control line 5\_0A were similar to those described on rice (Kuwata, 1985), with the appearance of halos around the inoculation site and the formation of brown/grey lesions (Fig. 4c). Symptoms were also comparable to those caused by *Pseudomonas syringae* pv. *coronafaciens* on its hosts, which is to be expected from their close phylogenetic relationship (Hwang *et al.*, 2005). We recovered about half the initial inoculum applied to the leaves at 0 dpi (Fig. S2a,b). In a time course experiment in which all plants were inoculated at 0dpi and samples taken every 24h, increasing numbers of bacteria were recovered from both wheat and barley, reaching a 100-fold increase at 3 dpi. Growth in barley appeared slightly higher than in wheat (Fig. S2a,b), corroborating the observations by Kuwata (1985) that it is easier to demonstrate growth in barley. Lesion size was positively correlated with the number of bacteria recovered from the corresponding leaf strip (Fig. S2c). To facilitate selection of Por36\_1 grown *in planta* and distinguish bacteria derived from our inoculum from contaminants among the colonies on plates inoculated with leaf extracts we generated rifampicin resistant mutants. Therefore, Por36\_1 was grown for 24 h at 28°C on KB agar (King *et al.*, 1954), bacteria were re-suspended in 5% KB liquid medium to an OD600nm = 0.01 and 100 µl where plated on KB plates with 15 mg l-1 rifampicin. Colonies from spontaneous mutants were re-streaked on KB plates with 50 mg l-1 rifampicin. Those growing within 24 h at 28°C were used for further characterisation by virulence assays on wheat line 5\_0A and barley cv Golden Promise as well as sequencing of 16S–23S rRNA internal transcribed spacer (ITS) region as described previously (Schoonbeek *et al.*, 2007). Of the isolates that had similar virulence and identical ITS to the original strain we picked one for all disease assays in this manuscript and named it Por36\_1rif.

**Notes S3** **Bioassays to determine reactive oxygen species (ROS) burst in response to PAMPS in wheat**

We tried to develop a luminol-based bioassay using protocols that work well in other species, such as *A. thaliana* (Felix *et al.*, 1999)*, Nicotiana benthamiana* (Lacombe *et al.*, 2010) or barley (Proels *et al.*, 2010). The following basic protocol was followed:

Leaf discs (d = 4 mm) were cut with a cork borer from the 2nd true leaf from 2-, 3- or 4-week-old wheat plants. The discs were incubated in 200 µl sterile water in a 96-well plate for 16h.The water was then drained and replaced by a solution containing 34 mg l‑1 (0.2 nM) 5-amino-2,3dihydro-1,4phtalazinedione (luminol), 20 mg l‑1 horseradish peroxidase, and the PAMP to be tested. The luminescence was recorded in 100 reads over an interval of approximately 40 minutes (Varioskan Flash plate reader; Thermo Fisher Scientific, Waltham, MA, USA). As variants on the basic protocol we either replaced the water with sodium phosphate buffer pH 7 or added 2 μg l‑1 Calyculin A (LC laboratories http://www.lclabs.com, Woburn, MA, USA), which increases responsiveness to PAMPs (Felix *et al.*, 1994) and was useful to measure PAMP responsiveness in Brassicas (Lloyd *et al.*, 2014). However, we never got reproducible induction of a ROS burst in any wheat plant, regardless of the PAMP and concentrations tested (100 to 1000 nM flg22, 100 to 1000 nM elf18, or 0.01 to 1 g l‑1 chitin), age of the plants at sampling (2-,3- or 4-week-old), or treatment with phosphate or Calyculin A. When using L-012 (Wako Chemical GmbH), a luminol derivative with increased sensitivity, we did not obtain a reproducible ROS burst without elevation of the background signal.

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