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The RNA-binding protein FPA regulates flg22-triggered defense responses and transcription factor activity by alternative polyadenylation

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RNA-binding proteins (RBPs) play an important role in plant host-microbe interactions. In this study, we show that the plant RBP known as FPA, which regulates 3′-end mRNA polyadenylation, negatively regulates basal resistance to bacterial pathogen *Pseudomonas syringae* in Arabidopsis. A custom microarray analysis reveals that flg22, a peptide derived from bacterial flagellins, induces expression of alternatively polyadenylated isoforms of mRNA encoding the defence-related transcriptional repressor ETHYLENE RESPONSE FACTOR 4 (ERF4), which is regulated by FPA. Flg22 induces expression of a novel isoform of ERF4 that lacks the ERF-associated amphiphilic repression (EAR) motif, while FPA inhibits this induction. The EAR-lacking isoform of ERF4 acts as a transcriptional activator *in vivo* and suppresses the flg22-dependent reactive oxygen species burst. We propose that *FPA* controls use of proximal polyadenylation sites of *ERF4*, which quantitatively limit the defence response output.

Plants are non-hosts to most pathogens due to pathogen associated molecular patterns (PAMP) triggered immunity (PTI). PTI is elicited through the PAMPs by extracellular pattern recognition receptors (PRRs) at the cell surface, resulting in widespread transcriptional reprogramming and broad-spectrum defence against potential pathogens. A well characterised PAMP-PRR interaction is the detection of the conserved 22-amino-acid peptide from bacteria, flg22, by the leucine-rich receptor kinase FLAGELLIN SENSITIVE2 (FLS2)[1,2].

Up to now, characterisation of the plant immune response has focused mostly on defining pathogen perception followed by activation of signalling cascades and rapid changes in transcriptional programmes. However, mechanism(s) by which the plant defence response is fine-tuned to avoid associated fitness costs is less well known. Emerging evidence indicates that regulation at the RNA level comprises another layer of regulation for both pathogen virulence and plant defence[3,4]. For instance, the RNA-binding protein (RBP) RBP35 is required for full virulence and development in the rice blast pathogen *Magnaporthe grisea*[5] whereas plant RBPs RBP-DR1 (RNA-BINDING PROTEIN-DEFENSE RELATED 1) and GRP7 (GLYCINE-RICH RNA-BINDING PROTEIN 7), positively regulate resistance to bacteria in Arabidopsis[6–9].

Alternative polyadenylation (APA) and splicing in plants are widespread: approximately 60–70% of all Arabidopsis genes are known to contain multiple poly(A) sites[10–12]. Widespread changes in APA are associated with mammalian cancerous cells[13,14] and differential poly(A) site choice has been observed in plants upon developmental changes or in response to salicylic acid treatment[11]. The Arabidopsis RBP FPA, a Spen family protein containing three RNA recognition motifs (RRMs), has been shown to regulate the 3′ end site choice at diverse mRNAs. The precise mechanism by which FPA regulates RNA 3′ end formation is unclear since FPA is not a known component of the conserved cleavage or polyadenylation apparatus[15,16]. FPA enables the transition

to flowering by repressing FLOWERING LOCUS C expression[17] but a role for this protein in a stress or plant defence response has not been shown.

Here, we show that FPA negatively regulates PTI responses such as the flg22-triggered ROS burst and bacterial accumulation in Arabidopsis. To see whether the role of FPA as a modulator of 3′ end RNA cleavage site choice contributes to the defence response mediated by FPA, we first searched for defence-related genes showing differential 3′ end RNA processing profiles in PTI. One such candidate was ETHYLENE RESPONSE FACTOR 4 (ERF4). ERFs are plant-specific transcription factors characterized by an APETALA2/ERF domain, which binds to DNA *cis* elements such as GCC boxes in target gene promoters[18] to regulate diverse biological processes. In Arabidopsis, the majority of the ERF proteins are transcriptional activators, but at least eight ERFs, including ERF4, possess an EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) motif that facilitates transcriptional repression[19].

Here, we show that FPA regulates *ERF4* poly(A) site choice, and importantly, that *ERF4* APA determines ERF4 activity. We report that ERF4 alternative polyadenylation is induced by the PAMP flg22 and that FPA inhibits this induction. Our results reveal a novel function for FPA in plant defense to bacteria and reveal a link between APA and plant immunity.

Results

The RNA-binding domain protein FPA negatively regulates PTI. Arabidopsis mutants defective in the RNA-binding domain protein FPA exhibit RNA 3′ misregulation[15]. Given that several RBPs have been shown to affect host-microbe interactions, we analysed *fpa* loss-of- function and FPA overexpressor lines for their response to biotic stress. We first asked whether these lines exhibit altered release of reactive oxygen species (ROS), which act as signalling messengers in response to diverse biotic and abiotic stimuli. *fpa* mutants displayed an enhanced flg22-triggered ROS burst, whereas this was diminished in plants overexpressing FPA (Fig. 1A). Next, we asked if *fpa* mutants showed an altered response to the bacterial pathogen *Pseudomonas syringae*, as an enhanced transient ROS burst has previously been reported to correlate with increased PTI[20,21]. Following either spray or syringe inoculation of *P. syringae* isolates, *35S::FPA* leaves accumulated 30- or 7-fold more bacteria than WT, respectively (Fig. 1B), suggesting that FPA can modulate basal plant defense in Arabidopsis.

Flg22 sensing triggers transcription of alternatively polyadenylated forms of the transcription factor ERF4. Since it is known that FPA modulates the 3′ end cleavage of RNAs, we searched for novel alternatively polyadenylated transcripts associated with PTI by examining the response of Arabidopsis seedlings to flg22, a well-characterized PAMP. For this purpose, we used a custom cDNA microarray based on TAIR8 annotation, containing approximately 6000 additional unannotated intergenic regions[22]. We compared the transcript profile of Arabidopsis wild type Col-0 (WT) seedlings with that of the flg22-insensitive receptor mutant *fls2* at 0, 15, 30 and 60 min after flg22 treatment. As expected, previously characterised PTI marker genes such as *WRKY29*, *WRKY11* and *MITOGEN-ACTIVATED PROTEIN KINASE 3*[23] were induced by flg22 in Col-0 but not *fls2* (**Table S1**). Two hundred and twenty-four probes corresponding to unannotated intergenic regions detected differentially expressed signals with >2 fold in Col-0 and <2 fold in *fls2* in at least two time points compared to the mock treatment. Among them, forty-two probes detecting high flg22 induction were selected and differential induction was quantified using quantitative RT-PCR (RT-qPCR) (**Table S2**). One of the most strongly induced signals corresponded to the probe *ATRIKEN28815*, which was chosen for further study (Fig. 2A). Rapid amplification of cDNA ends (RACE) was used to detect the full-length cDNA corresponding to *ATRIKEN28815* and identified two transcripts (2445 nt and 1121 nt in length), which defined previously undetected alternatively polyadenylated isoforms of *ERF4* mRNA (Figs. 2B **and S1**).

The 5′ end of these newly detected *ERF4* mRNA isoforms map to the 5′ untranslated region (UTR) of *ERF4*, while the 3′ end maps to an intergenic region 1304 nt downstream of *ERF4* (as annotated in the most recent Arabidopsis genome release, TAIR10) (Fig. 2B). These experiments showed that the two distally polyadenylated *ERF4* transcripts differ by the presence of an intron, the excision of which removes a sequence encoding the *ERF4* EAR motif (Figs. 2B, 2C, **S1**). Hereafter, we refer to the annotated *ERF4* as *ERF4-R* (for repressor), the new longer transcript as *ERF4-IR* (for intron retention) and the new shorter transcript as *ERF4-A* (for activator, see below; Fig. 2B).

To quantify the *ERF4* APA levels, we designed RT-qPCR primers to specifically amplify the two alternatively polyadenylated *ERF4* isoforms, *ERF4-A* and *ERF4-IR*. Since *ERF4-IR* and *ERF4-R* sequences overlap, *ERF4-R* primers amplify both *ERF4-R* and *ERF4-IR* isoforms (Fig. 2B). We characterised in detail expression of *ERF4* isoforms in response to flg22. *ERF4-R* was detectable by RT-qPCR with or without flg22 treatment in both *fls2* and Col-0 seedlings, but *ERF4-IR* and *ERF4-A* were only observed 15–30 min or 30–60 min, respectively, after flg22 treatment of WT seedlings (Fig. 2D). This RNA processing response was transient, as *ERF4* APA transcript levels returned to almost basal levels by 3–6 h post-flg22 treatment (Fig. 2E). *ERF4-R* was induced slightly in *fls2*, but neither *ERF4-A or ERF4-IR* expression was induced in *fls2* at any time point, demonstrating the requirement of flg22 sensing for the induction of *ERF4* APA. To determine if the newly identified alternatively polyadenylated *ERF4* isoforms were also found in response to other biotic stress-related treatments, we tested expression of *ERF4-R*, *ERF4-IR* and *ERF4-A* in response to defence hormones salicylic acid or methyl jasmonate (MeJA). Although *ERF4-A* was strongly induced by flg22, neither SA nor MeJA significantly altered *ERF4-A* expression (Fig. S2), suggesting that *ERF4* APA is a specific response that occurs in the early stage of PTI.

FPA affects *ERF4* RNA procesing. Using direct RNA sequencing (DRS), we detected an increased abundance of distally polyadenylated transcripts corresponding to *ERF4-A* and *ERF4-IR* in *fpa-7* mutants, suggesting that FPA negatively regulates *ERF4* APA (Fig. 3A). The 3′ end of the *ERF4* APA isoforms is cleaved in intergenic sequence with upstream canonical poly(A) *cis* elements (AAUAAA -19 nucleotides upstream of the cleavage site and a U-rich sequence immediately upstream of the cleavage site) previously shown to be associated with preferred cleavage sites in Arabidopsis 3′UTRs[12], whereas canonical poly(A) *cis* elements could not be detected at the 3′ end of the annotated *ERF4* isoform (Figs. S1, 3B).

Importantly, we found that the alternatively polyadenylated isoforms *ERF4-A* and *ERF4-IR* were upregulated in both *fpa* mutant alleles relative to Col-0, with *ERF4-A* expressed >60 fold more in independent *fpa* mutant alleles compared to WT plants. In contrast, transgenic plants overexpressing FPA exhibited reduced *ERF4-A* expression (6 fold lower than WT), but showed WT levels of *ERF4-IR* expression (Fig. 3C). Together, these data indicate that FPA negatively regulates the expression of *ERF4-A*. Since the Arabidopsis flowering time regulator FCA can also affect RNA 3′ end formation[15], we analysed *ERF4* RNA processing in plants defective in FCA or FLD, a histone demethylase which also regulates flowering time in Arabidopsis through the same pathway[24]. Neither *fca-9* nor *fld-3* exhibited altered *ERF4* poly(A) site selection or *ERF4-A* expression relative to WT plants. Furthermore, *ERF4* poly(A) site selection was similar in *fpa-7 fca-9* double and *fpa-7* single mutants, revealing that FCA does not act redundantly with

FPA in *ERF4* APA control and that late flowering and elevated FLC levels do not indirectly alter ERF4 APA (Figs. 3A, S3).

APA of *ERF4* generates new functional specificities. We reasoned that ERF4-A might lack transcription repressor activity due to loss of the EAR motif. To test this hypothesis, we analysed expression of the luciferase (LUC)-encoding reporter gene fused to a GCC box promoter using a transient transcriptional activity assay. When introduced by bombardment into Arabidopsis cells the *ERF4-R* coding region reduced the expression of the reporter gene >2-fold, whereas expression of the *ERF4-A* coding region activated the expression of the reporter gene >7-fold (Fig. 4A). These data indicate that deletion of the EAR motif, caused by alternative processing of *ERF4* pre-mRNA in response to flg22 perception, switches ERF4 from being a transcriptional repressor to an activator.

To assess potential biological roles of ERF4-A and ERF4-R *in planta*, we generated transgenic Arabidopsis lines stably overexpressing the same *ERF4* isoforms as those used in the transient assays. The multiple lines highly expressing *ERF4-A* under the control of the *35S* promoter were larger than WT plants, whereas the majority of the *35S::ERF4-R::nos* transformants were stunted when grown under long day growth conditions (Fig. 4B). The contrasting phenotypes of these lines are consistent with the idea that alternatively polyadenylated *ERF4* isoforms encode proteins with distinct *in vivo* functions.

To understand the individual biological roles of ERF4-A and ERF4-R in plant defence, we transformed the T-DNA insertion line

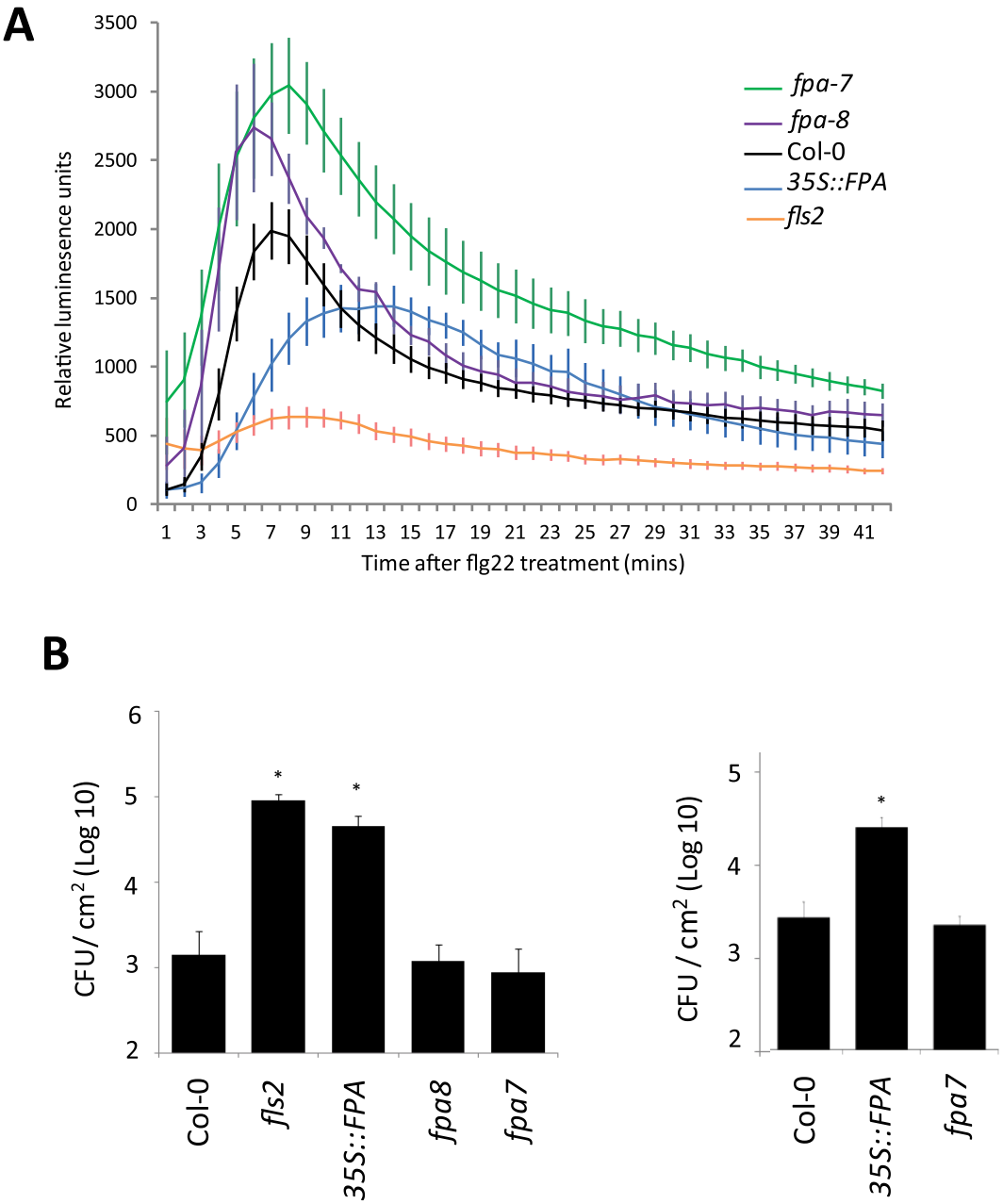

Figure 1 | FPA negatively regulates plant immunity. (A) Oxidative burst elicited by flg22 (1 μM) in Col-0, *fls2*, *35S::FPA*, *fpa-7* and *fpa-8* seedlings. Mean data with standard error from 3 biological replicates are shown. (B) Number of bacteria extracted from leaves of plants 3 days post-spray inoculation with *Pst*DC3000Δ*avrPto*Δ*avrPtoB* (OD_{600} = 0.2; left panel) or 3 days post-syringe infiltration with *Pst*DC3000 (OD_{600} = 0.002; right panel). Data shown are the mean colony forming units (CFU)/cm^2 extracted from leaf discs from five plants per genotype. Standard errors are shown. Asterisks indicate a difference from Col-0 ($P < 0.05$).

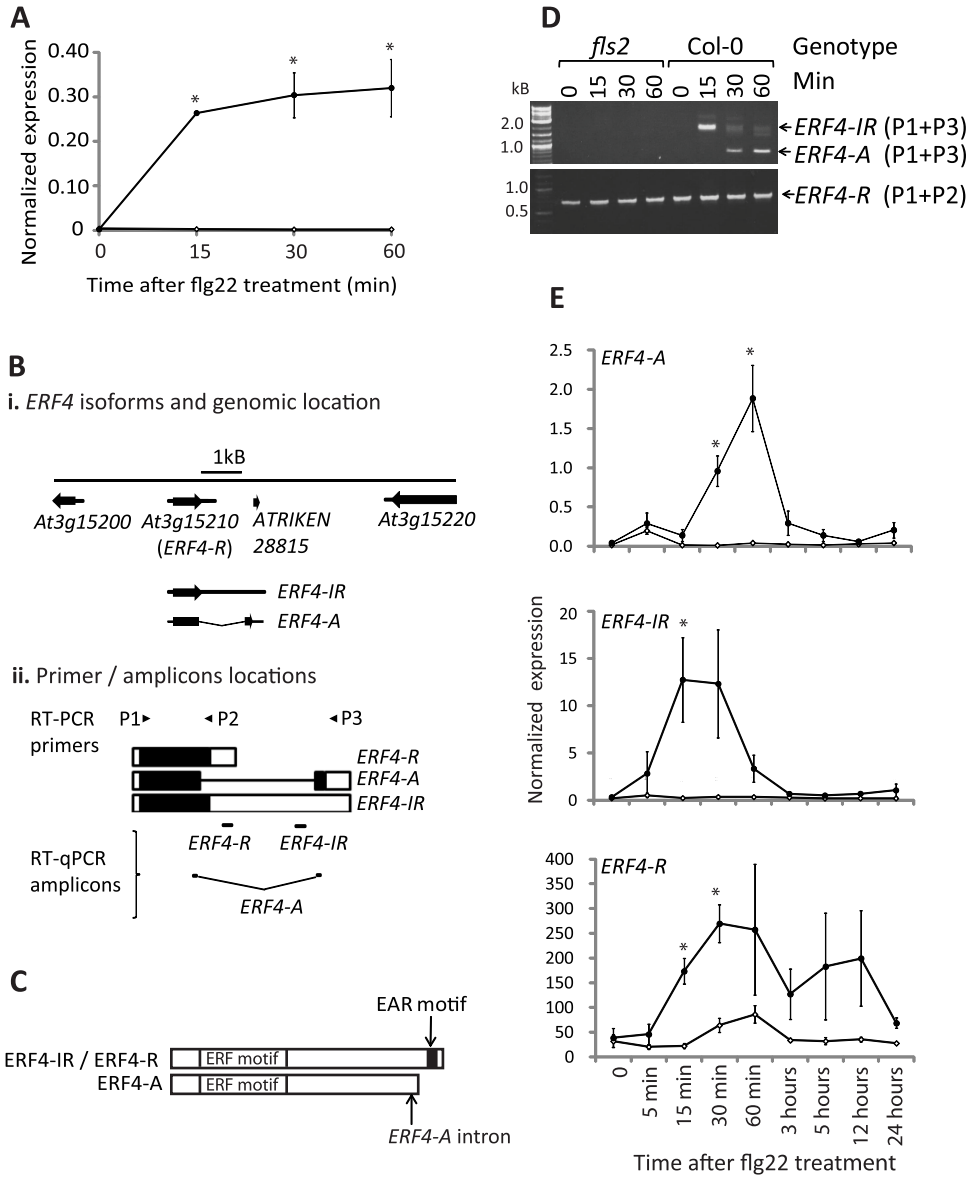

Figure 2 | Alternatively polyadenylated and spliced isoforms of ERF4 are rapidly and transiently induced by flg22 treatment. (A) Quantitative RT-PCR (RT-qPCR) validation of *ATRIKEN28815* expression in Col-0 (filled circle) and *fls2* (open circle) seedlings following treatment with 1 μM flg22. Data show the mean and standard error of two biological replicates. Asterisks indicate differences between Col-0 and *fls2* ($P < 0.05$). (B) i) Schematic diagram of a 7.5-kB genomic fragment, indicating annotated *ERF4* (*ERF4-R*), *ATRIKEN28815* probe and flanking gene locations. Full-length cDNAs corresponding to *ERF4-A* and *ERF4-IR* are indicated below the annotated genes. Thick arrowheads denote exons, thicker lines denote UTRs and thin lines denote introns. ii) Primers used for transcriptional analyses. Conventional RT-PCR primers used in figure (D) are indicated by arrowheads, RT-qPCR amplicons used in (E) are indicated by thick lines and thin lines indicate the intron present in the *ERF4-A* amplicon. (C) The predicted *ERF4-A* coding region lacks the EAR motif present in both ERF4-R and ERF4-IR. (D) Conventional RT-PCR using primers P1 and P3 (top panel; 35 cycles) or P1 and P2 (bottom panel; 30 cycles) shows that the distally polyadenylated intron-retaining isoform *ERF4-IR* is rapidly induced within 15 min, followed by induction of the distally polyadenylated spliced isoform *ERF4-A* after 30 min. (E) RT-qPCR analyses of *ERF4* isoforms in Col-0 or *fls2* seedlings following treatment with water (0 time point) or 1 μM flg22. Asterisks indicate differences between Col-0 and *fls2* ($P < 0.05$). Respective amplicons are shown in (B).

erf4[25] with either the *ERF4-R* or *ERF4-A* isoform under the control of the native *ERF4* promoter (referred to as *erf4*/*ERF4-R* and *erf4*/*ERF4-A*; Figs. 5A and S4). We first asked whether these lines exhibit altered release of flg22-dependent ROS. Independent *erf4*/*ERF4-A* and *35S::ERF4-A::nos* lines exhibited reduced ROS bursts relative to WT and *erf4* whereas independent *erf4*/*ERF4-R* and *35S::ERF4-R::nos* lines exhibited enhanced ROS bursts relative to WT (Fig. 5B).

We next asked how the expression of *PDF1.2*, which encodes a defensin involved in the jasmonate defence signalling pathway is affected by different *ERF4* isoforms. ERF4-R was previously shown to be a negative regulator of *PDF1.2*[25,26]. Indeed, *35S::ERF4-R::nos* and *erf4*/*ERF4-R* seedlings showed reduced basal *PDF1.2* expression relative to WT whereas *erf4* and *35S::ERF4-A::nos* lines exhibited enhanced basal *PDF1.2* expression relative to WT (Fig. 5C). These data reveal that *ERF4-A* and *ERF4-R* isoforms have opposing roles in regulating both the ROS burst and *PDF1.2* expression. However, *erf4*, as well as complementation and overexpression lines, responded similarly to WT plants when inoculated with the bacterial pathogen *P. syringae* under our experimental conditions (Fig. S5).

Flg22-triggered induction of *ERF4-A is* inhibited by FPA. We next examined whether the FPA- and flg22-dependent control of *ERF4*

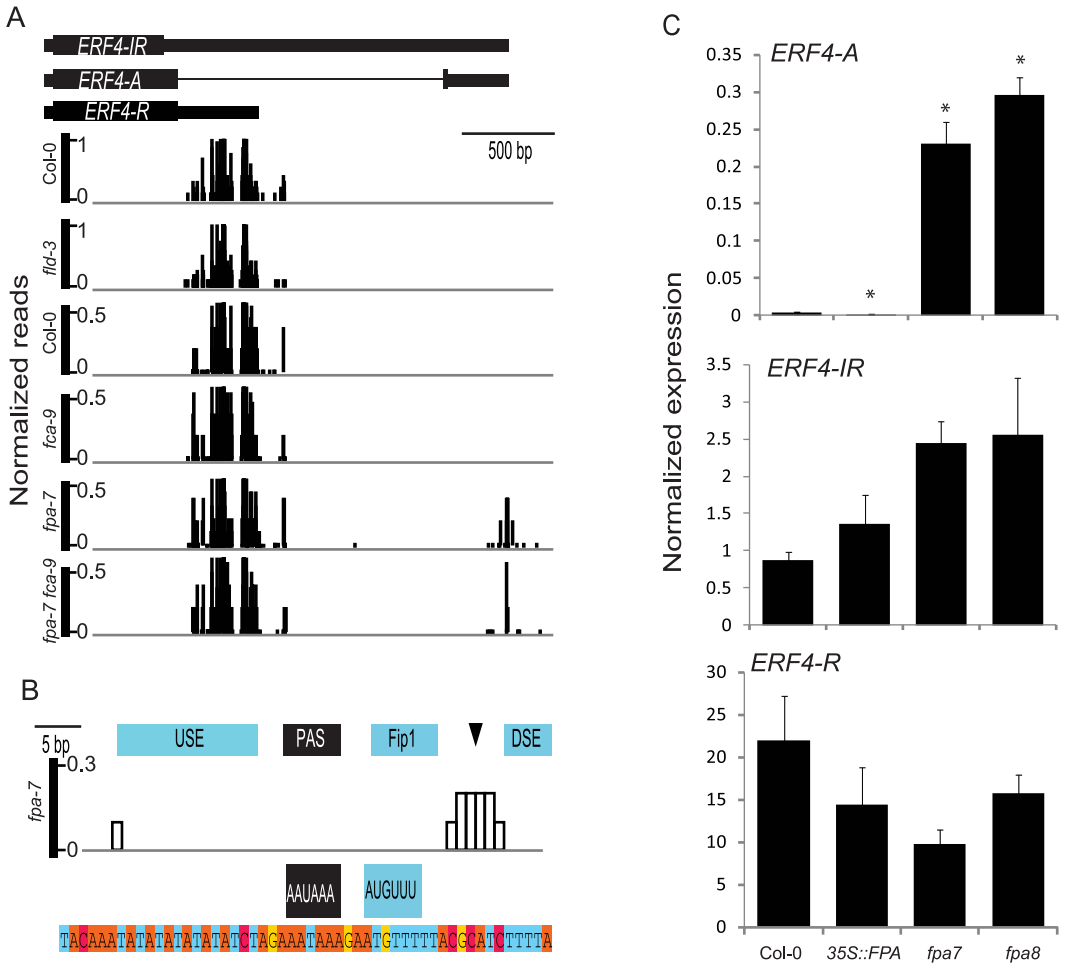

Figure 3 | Use of the *ERF4* distal polyadenylation site is increased in *fpa* plants. (A) Distribution of direct RNA sequencing (DRS) normalized reads at the *ERF4* locus. DRS was performed on RNAs from 14-day-old seedlings of Col-0 and *fca-9*, *fpa-7* and *fpa-7 fca-9* flowering time mutants for the first experiment and of Col-0 and the *fld-3* flowering time mutant for the second experiment. Representative Col-0 data for each experiment is shown above the mutant data. Exons are denoted by rectangles and UTRs by adjoining narrower rectangles. (B) Proposed designation of alternating U- and A-rich sequences at the *ERF4-A* or *ERF4-IR* downstream cleavage sites. Location of the AAUAAA and AUGUUU *cis* elements (AUGUUU corresponds to the point mutation in the UUGUUU motif) are displayed, as are their positions relative to the cleavage site. USE, upstream sequence element; PAS, polyA signal; Fip1, the U-rich sequence upstream of the cleavage site is the proposed Fip1-binding site[56,57]; DSE, downstream sequence element; black triangle, cleavage site. (C) Expression of the *ERF4* amplicons indicated in Fig. 2A in Col-0, *35S::FPA*, *fpa-7* and *fpa-8*. Data represent the mean and standard error of three biological replicates. The asterisk indicates a difference from Col-0 ($P < 0.05$).

APA might be mediated through a single signalling pathway. Basal expression of *ERF4* APA transcripts was increased in independent *fpa* mutant alleles, whereas *35S::FPA* plants exhibited a reduction in *ERF4* APA transcripts relative to WT (Figs. 3C, 6A). Since flg22 and FPA differentially modulate poly(A) site usage at *ERF4*, we speculated that disruption of FPA function might account for increased *ERF4* readthrough upon flg22 treatment. In such a scenario, flg22-triggered induction of *ERF4* APA would be abolished in *fpa* mutants. However, on the contrary, *ERF4* APA isoforms increased both in WT and *fpa* mutants after flg22 treatment (Fig. 6A). These data indicate the possibility that genetically distinct pathways converge to regulate *ERF4* RNA processing. *ERF4-R* remained the predominant *ERF4* isoform in all genotypes, either before or after flg22 treatment (Figs. 2E, 6A), suggesting that increased *ERF4* APA isoforms in *fpa* mutants or in flg22 treated plants cannot be explained solely as a consequence of a binary switch in poly(A) site use. Together, these data suggest that flg22 promotes use of the canonical distal poly(A) site, possibly by inhibiting use of the proximal poly(A) site to facilitate readthrough, resulting in increased expression of the *ERF4-A* isoform. On the other hand, FPA, at least partially independently of flg22, may promote use of the proximal poly(A) site, resulting in *ERF4-R* expression and inhibiting readthrough (Fig. 6B).

Discussion

Emerging evidence indicates that RNA processing is part of an active defense response in plants[27]. APA has previously been implicated as a modulator of plant immune responses mainly because mutations in genes involved in APA or RNA processing show altered defense responses and disease phenotypes[7,8,28]. In most cases, how poly(A) site choice is regulated during plant defense is not known. In this study, we uncovered a novel mechanism by which transcription factor activity may be regulated as a consequence of FPA mediated 3′ end RNA processing and alternative splicing.

FPA contains a RRM but the precise mechanism by which FPA controls 3′ end formation (and possibly other processes such as splicing) of RNA targets is unclear. FPA controls polyadenylation of its own transcript and of others by promoting polyadenylation at the proximal site, thereby inhibiting transcriptional readthrough to a stronger distal poly(A) *cis* element[16]. At *ERF4*, an identified ultimate target of FPA, intergenic distal poly(A) *cis* elements are canonical,

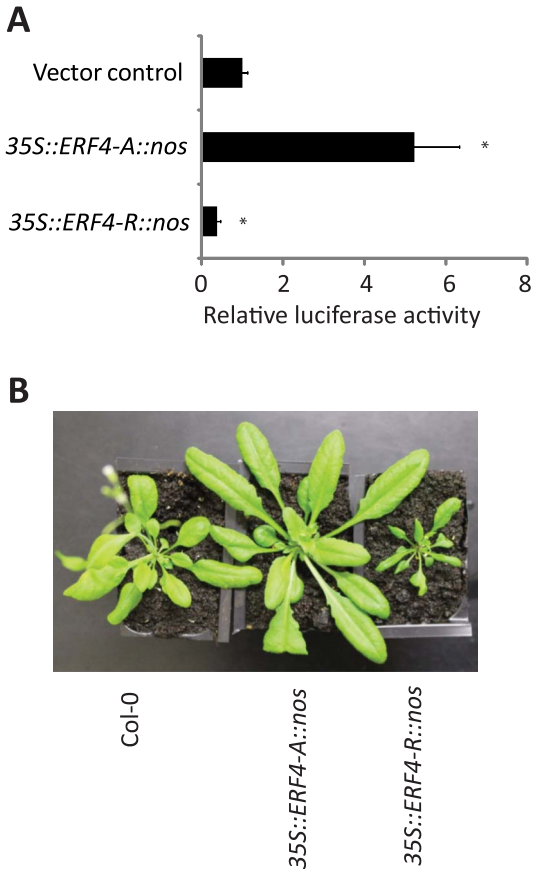

Figure 4 | Overexpression of *ERF4-R* or *ERF4-A* produce contrasting phenotypes. (A) Relative luciferase activities after cobombardment of *Arabidopsis* suspension cells with the GAL4GCC-LUC reporter gene. Data represent the mean and standard error of three biological replicates. Asterisks indicate differences from the empty vector control ($P < 0.05$). (B) Representative 6-week-old plants grown under long day conditions showing the morphological effects of *ERF4-A* and *ERF4-R* ORF overexpression.

suggesting a strong poly(A) site that does not depend on FPA function. Since the expression of the proximally polyadenylated isoform *ERF4-R* accumulated to higher levels than *ERF4* APA transcripts *ERF4-A* or *ERF4-IR*, even in the absence of a functional copy of FPA, proteins with partial functional redundancy to FPA, or more generic cleavage and poly(A) complexes are likely to promote proximal poly(A) site use at *ERF4*. FCA has been shown to act synergistically with FPA to control 3′ ends at several loci[15], however neither *fca* nor *fld* loss-of-function mutants[24] exhibited altered RNA processing at *ERF4*.

Loss of function *fpa* mutants exhibit defective 3′ end formation and consequent intergenic read-through at specific loci[15,16]. We show here that such read-through events are not necessarily benign. Read-through is often associated with cryptic splicing events that are not normally detected in WT[15]. We demonstrate here that such read-through coupled with cryptic splicing can alter the coding sequence and function of the upstream gene. These otherwise unpredicted consequent changes in RNA processing therefore have generally important implications for understanding the impact of APA and defective RNA 3′ end formation on gene regulation and disease[29].

In this study, we showed that APA of *ERF4* is of biological relevance since it results in generation of a new ERF4 isoform that lacks the well characterized EAR repression motif of this transcription factor. Indeed, using *in vivo* experiments we showed that this new ERF4 isoform, named as ERF4-A, acts as a transcriptional activator. Mechanisms such as phosphorylation[30] or proteolysis[31] have been shown or proposed to regulate the activity of ERF proteins. Our results show that APA-mediated EAR motif deletion is a novel mechanism by which ERF transcription factor activity can be manipulated. *ERF4* transcripts are highly unstable[32], suggesting a high turnover of mRNA whose polyA site usage can be regulated depending on the signalling requirements of the plant. We propose that *ERF4-A* acts to dampen the amplitude of the ROS burst, thereby preventing a 'run-away' defence response.

Intriguingly, when expressed in transgenic plants, ERF4-A acts as a negative regulator of flg22-triggered ROS burst. Therefore, one important function of ERF4-A generated by flg22 might be to control ROS rapidly generated in plant cells upon perception of flg22, so that potentially detrimental effects of these important signalling (but also damaging) molecules on plant cells can be minimized. Direct downstream targets of ERF4 are still largely unknown, but *PDF1.2*, encoding a plant defensin with antimicrobial properties, has been shown to be repressed by ERF4[25]. As expected, *PDF1.2* was differentially expressed in transgenic plants expressing *ERF4-A* or *ERF4-R*. OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF-domain protein 59 (ORA59), ERF104 and ERF1 have been shown to bind directly to the promoter of *PDF1.2*[33–35], but to date, there is no experimental evidence showing that ERF4 binds directly to the *PDF1.2* promoter. Therefore ERF4 may act upstream or in competition with these ERFs to fine tune plant defense. When grown under long day conditions, overexpression of *ERF4-R* or *ERF4-A* resulted in T_1 stunted or healthy plants, respectively, In contrast, McGrath *et al.* (2005) reported that *35S::ERF4* plants exhibited a WT growth morphology. Reasons for this discrepancy may include variations in environmental growth conditions or the constructs used to transform plants. While we transformed plants with the At3g15210 exon sequence, constructs used by McGrath *et al.* (2005) included regions of the At3g15210 3′ and 5′ UTRs.

ERF4 interacts with several corepressor proteins including TOPLESS (TPL), TPL-related (TPR), SIN3 ASSOCIATED POLYPEPTIDE 18 and ARABIDOPSIS HISTONE DEACETYLASE 19[36,37], which recruit chromatin modifying enzymes required for transcriptional suppression[38,39]. TOPLESS has been shown to bind directly to the EAR motif in several protein-protein interactions[40,41], suggesting that the ERF4-TOPLESS interaction might be EAR-motif dependent. Given that ERF4-A and ERF4-R differ by the presence or absence of the EAR motif, EAR-motif dependent protein interactions could result in differential function or even subcellular localization of ERF4-R and ER4-A. It is also possible that *ERF4* isoforms compete for the same DNA binding site or heterodimerize *in planta*.

Interestingly, altered APA at *ERF4* in *fpa* could not account for the enhanced defence phenotype in *fpa*. Since intergenic regions downstream of several loci are upregulated in *fpa*[15], ERF4 is likely to be one of several defence genes whose RNA processing is regulated by FPA. Furthermore, flg22-triggered modulation of APA may not be specific to *ERF4*. Identification of the defence-associated gene(s) or factors which are modulated by FPA and upon biotic stress requires further investigation. It is likely that APA at multiple loci in *fpa* could contribute quantitatively to achieve an overall positive regulation of PTI (Fig. 7). Plants are sessile organisms and are exposed to an abundance of microbes. While some microbes that come into contact with the plant are pathogenic, the majority are likely to be non-pathogenic. PTI is therefore under tight negative regulation to avoid expending energy on defence when it is not required. APA is likely to be an important process that allows the plant to rapidly expand the complexity of the transcriptome and/or proteome in response to flg22 and other stresses, but must be kept 'under wraps' to avoid an overreaction of the plant.

In addition to its role in defence regulation, FPA also promotes the transition to flowering. Interestingly, another Arabidopsis RBP,

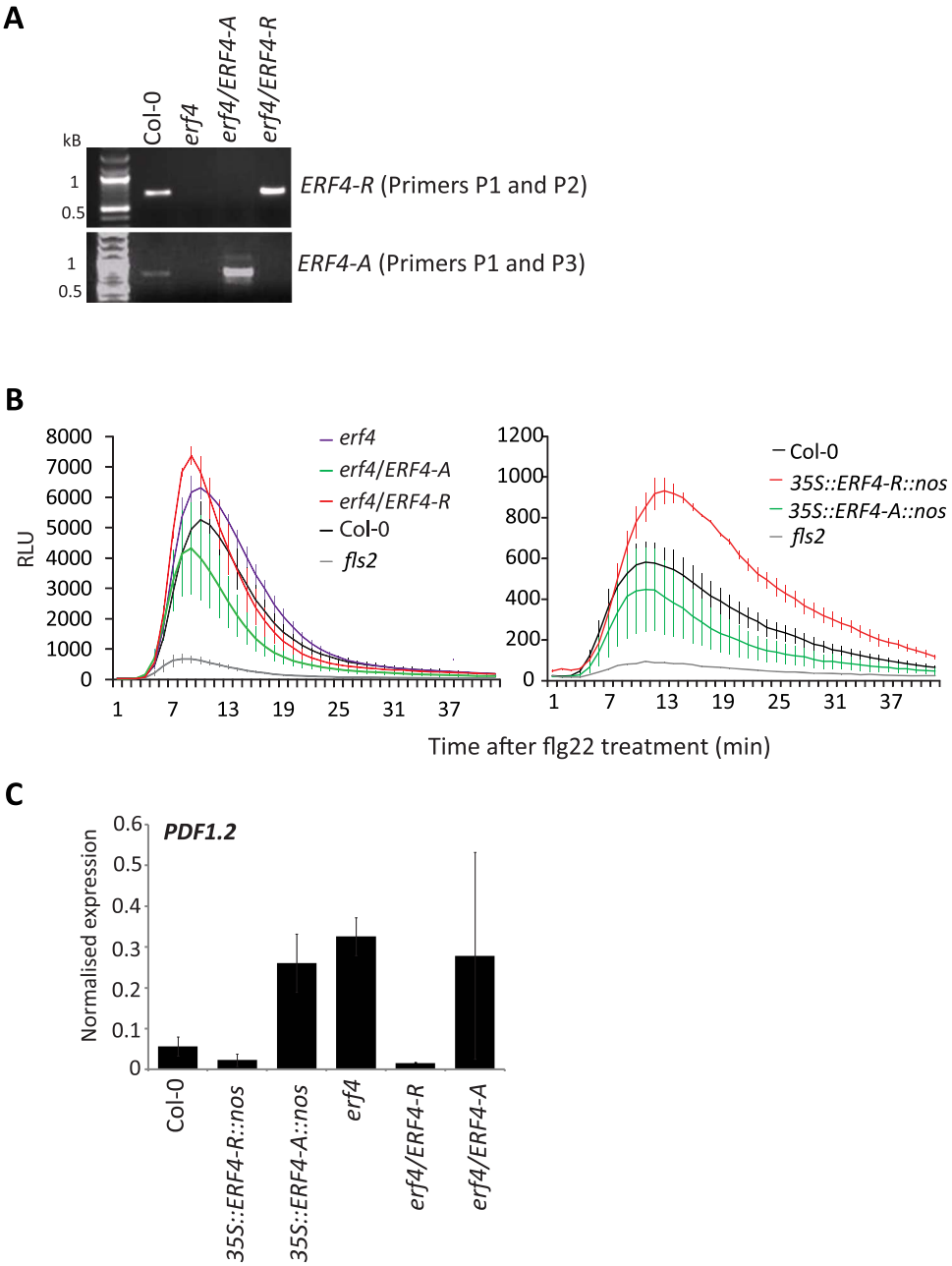

Figure 5 | *ERF4* isoforms differentially regulate both flg22-triggered ROS burst and *PDF1.2* expression. (A) RT-PCR showing the presence or absence of *ERF4* isoforms 30 min after 1 μM flg22 treatment of Col-0, *erf4*, *erf4/ERF4-A* and *erf4/ERF4-R* seedlings. (B) The oxidative burst elicited by 1 μM flg22 in representative *ERF4* isoform complementation (left) and overexpression (right) lines. Data represent the mean and standard error of three biological replicates. (C). Expression of *PDF1.2* in 14-day-old seedlings of the indicated genotypes. Data represent the mean and standard error of three biological replicates.

GRP7, also promotes the transition to flowering. In contrast to FPA, which negatively regulates PTI, GRP7 positively regulates PTI[42]. Plants need to maintain a tradeoff between development and defense to ensure optimal reproductive outcomes while surviving biotic and other stresses[43]. The interplay between flowering and defence has demonstrated by several examples in other pathosystems. Known mediators of both flowering and bacterial defence are PLANT U BOX PROTEIN 13 [44], HOPW1-1-INTERACTING 3 [45], LEAFY[46], SIZ1[47] and ENHANCED DOWNY MILDEW 2 [48].

In summary, the findings we present here may reflect the independent regulation by FPA and other RBPs of different targets involved in defence and flowering. Alternatively, such interactions may define a facet of the integration of these crucial life history traits, thus linking flowering with immunity at the molecular level.

Methods

Plant material, growth conditions and flg22 treatment. Arabidopsis plants were grown at 22°C under a 16 h light: 8 h dark cycle (normal day length conditions) or an 8 h light: 16 h dark cycle (short day length conditions). The WT genotype used was Col-0. *erf4*, *fpa-7*, *fpa-8* and *35S::FPA* lines were described previously[16,25,49]. Seedling treatment with flg22 was performed as described previously[23]. Briefly, stratified Arabidopsis Col-0 and *fls2* seeds were grown on plates containing 1× Murashige and Skoog medium (MS), 1% sucrose and 0.8% agar under normal day length conditions for 12 days. Seedlings were then transferred to liquid MS containing 1 μM flg22 or water for 2 days before harvesting. The flg22 peptide was purchased from Sigma and solubilized in water.

Flg22-triggered ROS burst. The flg22-triggered ROS burst was assayed as described previously[50]. Ten seeds of each genotype were dispensed per well, in triplicate, into a 48-well plate (NUNC). After surface sterilization, MGRL nutrient medium[51] supplemented with 0.1% sucrose was added to the plate and seeds were stratified for 2 days at 4°C. After 8 days growth under normal day length conditions, liquid medium

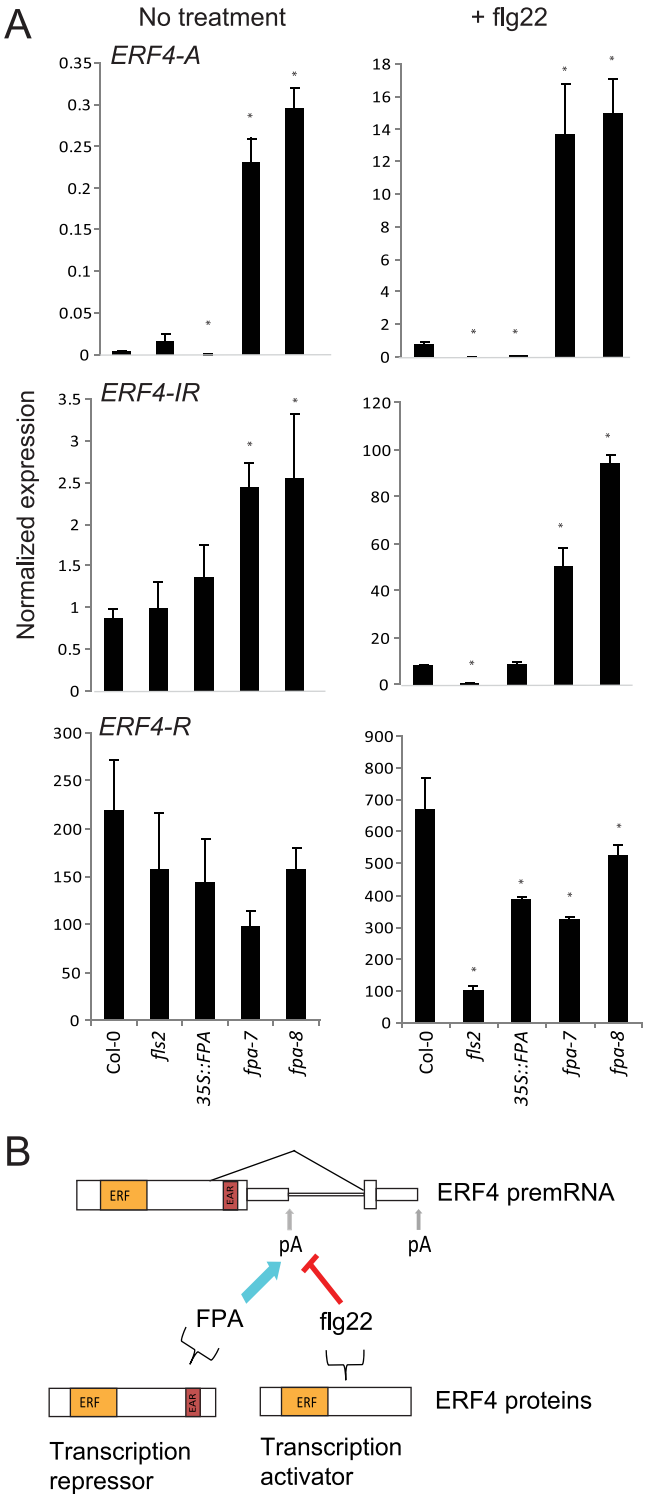

Figure 6 | FPA inhibits flg22-triggered induction of *ERF4-A*. (A) Expression of the *ERF4* amplicons indicated in Fig. 2B in Col-0, *fls2*, *35S::FPA*, *fpa-7* and *fpa-8* seedlings either untreated (left) or treated with 1 μM flg22 for 1 h (right). Data represent the mean and standard error of three biological replicates. The asterisk indicates difference from Col-0 ($P < 0.05$). (B) Schematic model indicating the role of flg22 and FPA in *ERF4* processing. FPA promotes use of the proximal poly(A) site, inhibiting readthrough to the canonical distal poly(A) site. flg22 promotes distal poly(A) site use, possibly by inhibiting proximal poly(A) site use, allowing readthrough and subsequent splicing.

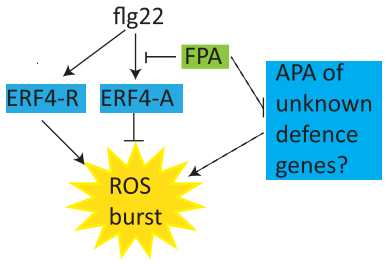

Figure 7 | Schematic model integrating the role of FPA and ERF4 isoforms in modulating the flg22-triggered ROS burst output. Upon flg22 treatment, the *ERF4* promoter is activated and expression of genes encoding both ERF4-R and ERF4-A, which positively and negatively regulate the ROS burst, respectively, increase. FPA partially inhibits the flg22-triggered induction of *ERF4-A* by promoting polyadenylation at *ERF4-R* (Fig S6). While ERF4-A suppresses the flg22-triggered ROS burst, FPA also suppresses the ROS burst. Therefore, APA modulation at ERF4 cannot explain increased ROS burst in *fpa* seedlings. It is possible that *ERF4* is one of multiple defence genes targeted by FPA under basal conditions or upon flg22 treatment in which altered APA, potentially leading to protein coding changes, results in the suppression of ROS production. We propose that such APA events are induced by flg22, but are inhibited by FPA to limit excess resource allocation to defense when it is not required.

was removed, replaced with MGRL nutrient medium supplemented with 0.1% sucrose and 100 μM L012 (Sigma) and plants were then incubated in the dark for 1.5 h. L012-containing medium was then removed and replaced with MGRL nutrient supplemented with 0.1% sucrose and 1 μM flg22. ROS production was measured immediately using a Mithras LB940 microplate luminometer (Berthold Technologies). At least 10 independent T_2 transformants for each complementation line and four independent T_2 transformants for each overexpression line were tested.

Bacterial spray pathotest. Six-week-old plants grown under short day conditions were sprayed with a bacterial suspension containing 1×10^8 cfu ml–1 PstDC3000ΔavrPtoΔavrPtoB[52] in 10 mM MgCl2 containing 0.04% Silwet L-77 (Bio Medical Science, Japan) and covered to maintain humidity. For syringe infiltration experiments, abaxial leaf surfaces were inoculated using a 1 mL needleless syringe with bacterial suspension containing 1×10^5 cfu ml^{-1}. Three days post-inoculation, counting of leaf bacteria by serial dilution plating was performed as previously described[53].

RNA isolation, microarray hybridization, RT-PCR and RT-qPCR. RNA was extracted and DNase treated using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg RNA using SuperScript RNA H- Reverse Transcriptase (Invitrogen) and oligo (dT) primer, according to the manufacturer's instructions. RT-PCR products were amplified in 25-μl PCR reactions containing 2 μl of first-strand cDNA, 1×PCR Buffer, 0.2 mM dNTPs, 0.5 U of Phusion DNA Polymerase (New England Biosciences) and 0.5 μM primer P1 and primer P2 or P3 (Table S3). RT-qPCR was performed using the Thunderbird SYBR qPCR mix (Toyobo, Japan) and 0.6 pmol primers (Table S1) according to the manufacturer's instructions using a Stratagene Mx3000P sequence detection system (Agilent Technologies). Quantities were determined against a standard curve using MxPro Software (Mx3000P version 4.1; Agilent Technologies) and normalized to actin. Plasmids containing equal molar ratios of *ERF4-A* or *ERF4-IR* were used to quantify *ERF4-A* and *ERF4-IR/ERF4-R* transcripts, respectively. All other genes were quantified using cDNA from Col-0 seedlings treated with flg22 for 30 min. All reactions were carried out in duplicate. RNA from each of eight samples (Col-0 or *fls2*: mock (0), 15, 30 and 60 min post-flg22 treatment) were reverse transcribed and labelled with Cy3 using the Quick Amp Labeling kit (Agilent), according to the manufacturer's instructions. Labelled cRNAs were then hybridized to RIKEN custom arrays as described previously[22].

Rapid amplification of cDNA ends. cDNA used for 3′ and 5′ RACE was prepared from 1 μg RNA extracted from Col-0 seedlings harvested 30 min after treatment with flg22. RACE was done using the GeneRacer kit (Invitrogen), according to the manufacturer's instructions and the gene-specific primers 28815_5′, 28815_5′ nest and 28815_3′ (Table S1). Five clones were sequenced per PCR product.

Dual-luciferase assay. To examine the transcriptional function of ERF4-R and ERF4-A, we performed a transient reporter assay using the Dual-Luciferase Reporter Assay System (Promega). To make effector constructs, the coding regions of ERF4-A and ERF4-R were amplified using ERF4_atg and ERFA_stop or ERF4R_stop primers, respectively (Table S1). The resulting *ERF4-R* and *ERF4-A* products were cloned into p35SNOSG. This vector contains the CaMV 35S promoter, a translational enhancer

from tobacco mosaic virus (Ω) and the GAL4GCC-LUC reporter gene. The effector, reporter and reference plasmids were introduced into Arabidopsis MM2D cell cultures by particle bombardment using a PDS-1000 particle gun (Bio-Rad). Luciferase activity was measured using a Mithras LB940 microplate luminometer (Berthold Technologies).

Generation of Arabidopsis transgenic plants. A modified *Agrobacterium tumefaciens*-mediated floral dip method[54] was used for plant transformation. To produce plants overexpressing ERF4-R or ERF4-A open reading frames (ORFs), effector plasmids used in the dual-luciferase assay were subcloned into the pBCKK vector and transformed into Col-0 plants. The growth morphology of 43 *35S::ERF4-R::nos* T_1 and 50 *35S::ERF4-A::nos* T_1 plants was assessed visually. To express *ERF4* isoforms under the native promoter in the *erf4* background, full-length cDNAs corresponding to ERF4 isoforms were amplified from cDNA using the primers ERF4_start and ERF4–3′_R (*ERF4-R* isoform) or ERF4_start and ERF4-3′_F (*ERF4-A* isoforms) and then fused to the native (−2401 nt) promoter amplified from gDNA using primers ERF4prom_F and ERF4prom_R (Table S1). Amplification products were cloned into pENTR (Invitrogen), subcloned into the promoterless pGWB1 vector[55] and then transformed into *erf4* plants.

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Author contributions

R.L., G.S., K.S., and K.S.* designed research; R.L., A.I., T.G., C.D., and M.H. performed research, K.H. and M.M. contributed new reagents/analytic tools; R.L., A.S., C.D., G.J.B. analyzed data; and R.L., K.K., K.S.* and G.S. wrote the paper.

Additional information

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