Phosphorylation-dependent control of an RNA granule-localized protein that fine-tunes defence gene expression at a post-transcriptional level

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SUMMARY

Mitogen-activated protein kinase (MAPK) cascades are key signalling modules of plant defence responses to pathogen-associated molecular patterns (PAMPs; e.g. the bacterial peptide flagellin (flg22)). Tandem zinc finger protein 9 (TZF9) is a RNA-binding protein that is phosphorylated by two PAMP-responsive MAPKs, MPK3 and MPK6. We mapped the major phosphosites in TZF9 and showed their importance for controlling in vitro RNA-binding activity, in vivo flg22-induced rapid disappearance of TZF9-labelled processing body-like structures and TZF9 protein turnover. Microarray analysis showed a strong discordance between transcriptome (total mRNA) and translatome (polysome-associated mRNA) in the tzf9 mutant, with more mRNAs associated with ribosomes in the absence of TZF9. This suggests that TZF9 may sequester and inhibit the translation of subsets of mRNAs. Fittingly, TZF9 physically interacts with poly(A)-binding protein 2 (PAB2), a hallmark constituent of stress granules – sites for stress-induced translational stalling/arrest. TZF9 even promotes the assembly of stress granules in the absence of stress. Hence, MAPKs may control defence gene expression post-transcriptionally through release from translation arrest within TZF9–PAB2-containing RNA granules or by perturbing the function of PAB2 in translation control (e.g. in the mRNA closed-loop model of translation).

Keywords: tandem zinc finger protein (TZF), processing bodies (PB), post-transcriptional regulation, pathogen-associated molecular pattern (PAMP), mitogen-activated protein kinase (MAPK), stress granule (SG).

INTRODUCTION

Plants are sessile organisms and are constantly exposed to a variety of potential pathogens in their environment. To combat these invaders, plants have developed both preformed and inducible defence systems. The first-tier of inducible defence, known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), is activated through recognition of conserved microbial features called PAMPs via membrane-localised receptors. A well-known PAMP is a 22-amino-acid long epitope of *Pseudomonas aeruginosa* flagellin (flg22). Flagellin is recognized by the FLS2 receptor (Gómez-Gómez and Boller, 2000), which triggers a complex network of downstream signalling pathways to initiate defence responses (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011; Bigeard et al., 2015). One of the central components of PTI includes phosphorylation-dependent activation of three-tiered mitogen-activated protein kinase (MAPK) cascades (Colcombet and Hirt, 2008). A typical MAPK cascade consists of a MAPK, a MAPK kinase (MAPKK or MKK) and an MKK kinase (MAPKKK or MEKK). The MAPKKs are serine or threonine kinases that phosphorylate downstream MKKs at conserved S/T-X3-X5-S/T motifs. Upon activation, MKKs in turn phosphorylate MAPKs at threonine or tyrosine residues in their activation loop (Suarez-Rodriguez et al., 2010). Activated MAPKs phosphorylate their substrate(s) at
unique (S/T)P sites (i.e. serine/threonine followed by a proline residue) (Pitzschke, 2015).

The flg22-responsive MAPks include MPK1, MPK3, MPK4, MPK6, MPK11 and MPK13 (Nühse et al., 2000; Zipfel et al., 2006; Bethke et al., 2012; Nitta et al., 2014). Among these, MPK3, MPK6 and MPK4 are the best studied and are known to alter the functions (e.g. stability or localisation) of their substrates after phosphorylation (Liu and Zhang, 2004; Lampard et al., 2008). Many of the MPK3 and MPK6 substrates regulate gene expression at the transcriptional level and there is increasing evidence for regulation at the post-transcriptional level (Lee et al., 2015). For example, the decapping activity of the mRNA decapping machinery is stimulated by the MPK6 substrate DCP1 (decapping protein 1) (Xu et al., 2006). Phosphorylation of DCP1 by MPK6 promotes interaction with DCP5 and thereby stimulates mRNA decapping under dehydration stress (Xu and Chua, 2012). Protein associated with topoisomerase II (PAT1), another enzyme from yeast and animal decapping complexes, also assists DCP2 in decapping and regulation of mRNA stability (Kulkarni et al., 2010). In Arabidopsis, PAT1 is phosphorylated by MPK4 after elicitation by flg22. This phosphorylation results in the re-localisation of PAT1 to cytoplasmic foci called processing bodies (PBs) for regulation of mRNA metabolism (Roux et al., 2015). Processing bodies are RNA–protein complexes found in the cytosol of eukaryotic cells. They control the decapping, degradation and storage of mRNA molecules and are usually visualized as speckle-like structures inside the cell (Kedersha et al., 2005; Parker and Sheth, 2007). Plant PBs have been shown to contain subunits of the decapping complex (DCP1, DCP2, DCP5 and VCS/Varicose), activators of the decapping complex such as Sm-like proteins (LSM1a and LSM1b), 5′→3′ exoribonuclease 4 (XRN4), deadenylation enzymes (PARN), miRNA-associated protein (Argonaute 1 or AGO1), nonsense-mediated mRNA decay (NMD) components (upframeshift proteins or UPFs) and tandem zinc finger proteins (TZFs) (reviewed in Maldonado-Bonilla, 2014). The Arabidopsis TZF protein family consists of 11 members with the so-called CCCH-type zinc fingers, where the zinc finger structure is stabilised by zinc coordinated by three cysteines and a histidine residue (Bogamuwa and Jang, 2014). Previously, we showed that the Arabidopsis tandem zinc finger 9 (TZF9) is a putative RNA-binding protein localised to PBs and is a phosphotarget of MPK3 and MPK6. Importantly, a tzf9 mutant showed attenuated early and late responses to PAMPs (Maldonado-Bonilla et al., 2014).

In this study, we re-validated the localisation of TZF9 in PB-like structures using electron microscopy and addressed its RNA-binding activity. We identified the major phosphorylated sites in TZF9 and showed that flg22-induced protein destabilisation, reduction of TZF9-labelled PB structures and RNA-binding properties are dependent on the phosphosites. Based on comparative transcriptomics and translatomics, both basal and flg22-responsive genes are deregulated and ‘translationally biased’ in the tzf9 background. TZF9 co-localizes and interacts with stress granule marker proteins such as the poly(A)-binding proteins (PABs). Together with the RNA-binding properties and localisation in RNA granule-like structures, our data suggest that TZF9 may regulate immunity through post-transcriptional processes such as translational control.

RESULTS

TZF9, a PB-localized protein, binds specific RNA sequences

When transiently expressed in protoplasts, TZF9–GFP fusion proteins are mainly in the cytoplasm with prominent cytoplasmic foci that co-localise with the PB marker DCP1 (Figure 1a). To increase the resolution of the localisation, we performed electron microscopy studies on stable transgenic 35S::TZF9-HA-YFP seedlings, which revealed clustered immune-gold labelling signals within spherical cytoplasmic PB-like structures (Figure 1b). In agreement with PBs as sites for mRNA processing or storage, RNA electrophoretic mobility shift assay (REMSA) was previously used to show binding of TZF9 to poly-rU (and weakly to poly-rG) RNA ribohomopolymers (Maldonado-Bonilla et al., 2014). To narrow down the binding specificities, we further performed an independent REMSA-based screen with a series of 12 RNA probes. These 90-nucleotide (nt)-long so-called ‘pentaprobes’ (Bendak et al., 2012) were designed to cover all 1024 possible 5-nt combinations (for the individual sequences see Figure S1a in the online Supporting Information) based on the assumption that binding motifs are typically at least 5 nt long. Strikingly, out of the 12 RNA probes, recombinant TZF9 bound only to pentaprobe-2, which has a base composition of U3/C1/G1/U1/A1 (Figures 1c and S1b), and can be considered to be U-rich (48% U).

We further employed a selective evolution of ligands by exponential enrichment (SELEX)-based strategy to enrich for TZF9-bound RNAs from a library of randomized 20-nt sequences (Figure S2a). For this, in vitro transcribed RNAs were incubated with recombinant TZF9 and the associating RNAs were isolated and reverse-transcribed into cDNA for cloning and sequencing (Figure S2b). Clones from the naïve library revealed only random sequences (not shown), while 18 sequences were associated with TZF9 and four with the CCCH-zinc-finger deleted TZF9 (Figure S2c,d).

Based on these limited sequences, four consensus motifs were deduced, of which three are U-containing probes (Figure 1d). The fourth motif, ‘G(C/A)G’, was also found to associate with the CCCH-deleted TZF9 variant (Figure S2d); however, since there was little to no detectable RNA recovered with the mutated TZF9 (Figure S2b), these four sequences obtained may be from random unspecifically bound RNAs. Thus, taken together, all the RNA-binding
analyses point to TZF9 binding to U-rich or U-containing sequences.

The major phosphorylation sites in TZF9 is from S^{181} to T^{377}

We reported previously that TZF9 is phosphorylated by MPK3 and MPK6 (Maldonado-Bonilla et al., 2014). TZF9 contains 14 (S/T)P motifs that are putative MAPK phosphosites (designated as PS-1 to PS-14; Figure 2a). To elucidate which of these are phospho-modified, we performed phosphosite mapping by mass spectrometry of in vitro MAPK-phosphorylated TZF9. Using MPK3 as the kinase, we identified five phosphopeptides corresponding to T377, S408, S415, S435 and S472 of TZF9 (Figures 2b and S3). Additionally, S418 is also phosphorylated; while not a typical MAPK-targeted site, it is adjacent to a classical (S/T)P site (PS-12). Hence, phosphosites were all mapped to the C-terminal half of TZF9, covering the sites designated as PS-9 to PS-14 (see Figure 2a).

As the mass spectrometry did not provide 100% coverage of TZF9, we alternatively tested the impact of individual phosphosites on phosphorylation. We generated PS mutant variants of TZF9 by mutating the Ser or Thr of the (S/T)P motif to non-phosphorylatable Ala or Gly by site-directed mutagenesis (Palm-Forster et al., 2012). Due to the proximity of some of the phosphosites, two of the generated constructs cover three mutated sites simultaneously (i.e. PS-6, -7, -8 and PS-10, -11, -12). Next, we performed radioactive in vitro kinase assays with these TZF9 variants as substrates and MPK3 (Figure 2c) or MPK6 (Figure 2d) as kinases. Several independent experiments were performed and the signal intensities of the phosphorylated TZF9 were quantified (Figure 2e,f). A reduced signal in PS-3 (S181A), PS-4 (S323A), PS-5 (S343G), PS-6, -7, -8 (T352A, S356A, S362A) and PS-9 (T377G) variants suggests that TZF9 can be phosphorylated at these sites by both MPK3 and MPK6 in vitro. Combined with the mass spectrometry data, TZF9 can be phosphorylated at multiple sites between PS-3 and PS-14 (Figure 2a), but the major sites contributing to the global phosphorylation level are probably PS-3 to PS-9 (Figure 2e,f).

Based on the above interpretation, three multisite phospho-mutants were generated: PS 3-5 (S181A, S323A, S343G), PS 6-9 (T352A, S356A, S362A, T377G) and PS 3-9
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(b) Table 1: MPK3-mediated phosphorylation sites identified by mass spectrometry

| Phosphorylation Site | Peptide Sequence | m/z     | Charge | Mascot Ion Score | Mascot Peptide Site Probabilities | PhosphoRS Peptide Site Probabilities |
|----------------------|------------------|---------|--------|-----------------|----------------------------------|-------------------------------------|
| T377                 | TWMWPNPTPPhPPALQLPGSR | 1130.040649 | 2      | 54              | T(9): 99.9%                      | T(9): 100%                          |
| S408                 | EIDFSEEMQSLTSPhPTTWNNTPMSSSPFSKG | 1110.141724 | 3      | 89              | S(13): 83.3%                     | S(13): 87%                          |
| T415, S418           | EIDFSEEMQSLTSPhPTTWNNTPMPhPSPhPSFSKG | 1126.79187 | 3      | 32              | T(20), S(23): 45.71% | S(13): 98.4%, S(23): 94.5%         |
| S435                 | LAGGAMS(PPh)PVSNLSMFGTEDNTSGLQIRR | 1035.472871 | 3      | 45              | S(7): 55.5%                      | S(7): 99.9%                         |
| S472                 | SVINPQHLSNLSSLSS(PPh)PVGANLSMDSDSSAVLSR | 1180.565674 | 3      | 34              | S(15): 35.11%                    | S(15): 95.3%                        |

(c) (d) (e) (f) (g) (h)
(S181A, S232A, S343G, T352A, S356A, S362A, T377G). Additionally, PS 1-14, carrying mutations of all 14 phosphosites, was also generated as a phospho-null control. These were transiently expressed in protoplasts as haemagglutinin (HA)-tagged proteins and the effect of flg22 treatment on the proteins was analysed by immunoblotting. As reported previously (Maldonado-Bonilla et al., 2014), TZF9 proteins extracted from flg22-treated protoplasts show a reduced mobility on SDS-PAGE (Figure 2g), which is abrogated by lambda-phosphatase treatment of the protein extracts (Figure 2h). Thus, this ‘mobility shift’ is due to in vivo phosphorylation and not other post-translation modifications. Based on comparison with the phosphatase-treated samples, it is also noteworthy that the native TZF9 is already partially phosphorylated prior to flg22 treatment; this is either basal phosphorylation or due to handling stress during protoplast preparation. The PS 3-5 and PS 6-9 variants showed additional partial mobility shifts upon flg22 treatment, suggesting that the mutated protein can still be phosphorylated. By contrast, the phospho-null and PS 3-9 variants showed no obvious mobility shift, even after treatment with lambda-phosphatase. Hence, these data suggest that the predominant sites for phospho-null treatment are the seven sites between PS-3 (S181) and PS-9 (T377). Furthermore, since the mobility shift can be mimicked by activating MPK3/6 (Figure S4) and abolished by mutating MAPK-targeted sites, MAPKs and not other flg22-responsive kinases are the major kinases relevant for in vivo phosphorylation after flg22 elicitation.

Localisation of TZF9 and protein stability change upon flg22 elicitation in a phosphorylation-dependent manner

To investigate if phosphorylation may alter the function of TZF9, we first looked at the cellular localisation of TZF9. The number of TZF9-labelled cytoplasmic PB-like foci differs substantially between cells, which makes it difficult to assess this globally. Therefore, to study the dynamics of the PB structures we used the ‘position tracking’ function of the confocal microscope to monitor each protoplast individually at different time-points after elicitation. For each time-lapsed record, images were scanned at multiple confocal Z-stacks (Figure S5a) to exclude that the PB particles have ‘drifted’ into a different focal plane. Figure 3(a) shows a representative protoplast individually, with the average results of multiple protoplasts (n = 8) graphically depicted next to it. Notably, the number of TZF9-GFP foci rapidly decreased within 4–16 min of flg22 treatment (Figures 3a and S5b). This depletion of PB structures was not observed with water treatment. Interestingly, no change was seen in the protoplasts expressing the PS 3-9 and PS 1-14 variants when treated with either flg22 or water, thus suggesting that the flg22-induced reduction of TZF9-labelled PB structures is dependent on these phosphosites.

Levels of TZF9 protein do not show obvious changes within the short duration of flg22 treatment (here, up to 16 min) (Figure S5c), so the disappearance of the PB structures is not due to depletion of TZF9 protein. However, longer elicitation periods have been reported to reduce TZF9 levels (Maldonado-Bonilla et al., 2014). To check if protein turnover is regulated by phosphorylation, we compared the protein stability of the phospho-mutants PS 3-9 and PS 1-14 with that of native TZF9. These were transiently expressed in protoplasts, which were subsequently treated with cycloheximide and flg22 simultaneously, and harvested at hourly intervals. Cycloheximide treatment was included to block de novo protein synthesis and therefore facilitate the visualisation of differences in protein turnover. Both the flg22-induced mobility shift and reduction in TZF9 protein levels can be seen starting at 1 h after treatment. By contrast, both of the tested phospho-mutants were comparatively more stable. Overall, the results indicate that flg22-induced phosphorylation (at MAPK-targeted sites) reduces the stability of TZF9. Additionally, an immunoreactive band corresponding to unphosphorylated TZF9 reappears at 2 h after the addition of flg22 (Figure 3b), thus suggesting that dephosphorylation of native TZF9 is occurring in the background. Thus, TZF9 turnover is regulated by phosphorylation.

Phosphorylation of TZF9 diminishes its RNA-binding capacity

The rapid flg22-induced disappearance of TZF9-labelled PB structures may reflect dissociation from bound RNAs. A possible controlling mechanism might be through the phosphorylation of TZF9. We thus used REMSA to compare the RNA-binding properties of native and phosphorylated TZF9. Phosphorylated TZF9 was prepared by incubation with recombinant MPK3 in the presence of non-radioactive ATP. Aliquots of the kinase-treated proteins were separated on SDS-PAGE and analysed by Pro-Q...
diamond phospho-staining (Figure 4a), which confirmed the success of TZF9 phosphorylation. In REMSA, phosphorylated TZF9 showed a strong reduction in binding to poly-U RNA probes (Figure 4b). Based on quantifications from triplicate experiments, the phospho-null version (PS 1-14) also showed a slight reduction in RNA binding (compared with the unmodified TZF9) but this is still higher than the phosphorylated TZF9 or the negative control (CCCH-

**Figure 3.** Localisation and stability of changes in tandem zinc finger protein 9 (TZF9) upon treatment with flagellin (flg22). (a) Images of TZF9 localised in cytoplasmic foci in protoplasts. Protoplasts expressing GFP-tagged TZF9-WT, TZF9-PS 3-9 or TZF9-PS 1-14 were imaged at the indicated time points after water (control) or flg22 treatment. Each row shows a representative protoplast, with the corresponding graphs (on the right) depicting quantification of foci in eight independent protoplasts (means ± SE). Scale bar = 10 µm; WT, wild type; PS, phosphorylation site. (b) Protoplasts expressing haemagglutinin (HA)-tagged TZF9-WT, TZF9-PS 3-9 and TZF9-PS 1-14 were treated with cycloheximide (5 µM) and flg22 (100 nM), and harvested at the indicated time points. We used α-HA blotting to display TZF9 protein levels and the 'phospho-mobility shift'. The loading control was monitored with amido-black staining of the blot.
deleted TZF9). In this case, although the phosphosite mutations are not within the zinc finger domains, the ternary protein structure may be affected and have an impact on RNA binding. Taken together, in vivo flg22-induced MPK3/6 activation and TZF9 phosphorylation can reduce RNA-binding by TZF9, which may explain the rapid disappearance of TZF9-labelled PB structures.

Transcriptome analysis shows that TZF9 is required for full responsiveness to flg22

The above findings of flg22-induced changes in TZF9 cellular localisation, protein stability and RNA-binding properties support a role for TZF9 in PTI signalling. This is in agreement with previous work showing attenuated PTI responses in the tzf9 mutant (Maldonado-Bonilla et al., 2014), where selected genes show diminished flg22-induced expression. To obtain a genome-wide overview, comparative microarray-based profiling of total mRNAs was performed. Leaves of wild-type (WT) Arabidopsis (ecotype Col-0) and tzf9 were infiltrated with 1 µM flg22 and harvested after 1 h (Figure 5a). As water infiltration can weakly induce MAPK activation and partial TZF9 phosphorylation, non-infiltrated leaves were used as a control (ctrl). Thus, note that the ‘flg22 response’ in our analysis includes infiltration stress. Total RNA from three independent biological replicates was processed for hybridization on an Affymetrix1.1 ST Exon Array Chip, of which about 50% (12 351 out of 24 000 genes) of the genes on the chip were detected. The data were filtered for differentially expressed genes (DEGs; false discovery rate-adjusted P < 0.05, log2 fold-change ±1) by comparing genotype and treatment, thus resulting in 2685 up- and 2103 downregulated DEGs (Table S1).

The DEGs are illustrated in a heatmap (designated as TC for the transcriptome dataset; Figure 5a) and numerically summarised in Figure 5(b) as a Venn diagram (comprising: Set 1, WT_flg22 versus WT_ctrl; Set 2, tzf9_flg22 versus tzf9_ctrl; Set 3, tzf9_ctrl versus WT_ctrl). If TZF9 is irrelevant for PTI, gene expression profiles would be similar between the WT and the tzf9 mutant. However, as illustrated by the top left sector of the Venn diagram (Figure 5b), 50% [(1122 + 51)/(1122 + 51 + 1102 + 59)] of the flg22-upregulated genes and 44% [(581 + 51)/(581 + 51 + 756 + 36)] of the flg22-downregulated genes in the WT plants are not flg22-responsive in the tzf9 background. Additionally, during real-time quantitative PCR validation of selected DEGs, the amplitude of the induction for some flg22-responsive genes was quantitatively reduced in the tzf9 background (Figure S6), which is also visible in the heatmap (Figure 5a). This confirms at the global gene expression level that flg22 responsiveness is compromised in the tzf9 mutant. Conversely, the top right sector of the Venn diagram (Figure 5b) also illustrates that in the absence of TZF9, a small set of genes (134 up- and 610 downregulated) ‘gained’ flg22-responsiveness, suggesting that TZF9 acts as a negative regulator for the expression of these genes in the WT background. Taken together, TZF9 acts mostly as a positive
The number of flg22-responsive genes in the tzf9 background is proportionally higher in the translatome

Since the RNA-binding properties and cellular localisation of TZF9 in PBs point to roles in post-transcriptional processes, we extended the transcriptome with a ‘translatome’ analysis. For this, actively translating ribosomes were immunoprecipitated from a transgenic line expressing the FLAG-epitope-tagged ribosomal protein L-18 under the control of the 35S promoter (35S::FLAG-RPL18) (Muströph et al., 2009). This transgene was introgressed into the tzf9 background through crossing. The experimental set-up for treatment and microarray was as for the transcriptome analysis except for the additional step of enriching for mRNAs from actively translating ribosomes (Figure 5a). To facilitate comparison, the DEGs were represented in the same manner as the transcriptome above as three sets in the Venn diagram (WT_flg22 versus WT_ctrl, tzf9_flg22 versus tzf9_ctrl and tzf9_flg22 versus WT_ctrl; Figure 5c, Table S2) and also combined into a single heatmap for both the transcriptome (TC) and the translatome (TL) (Figure 5a, right panel).

Comparing the total number of DEGs, there are about a thousand more downregulated genes in the translatome than in the transcriptome (i.e. 3055 versus 2103). While this may be caused by differential sensitivities of the two mRNA isolation/enrichment methods, the bulk of this difference is reflected in the large number of flg22-repressed genes (i.e. 1363 compared with 607 in the transcriptome) exclusive to the tzf9 background (Figure 5c). Conversely, while the total number of flg22 upregulated genes is quite similar between the transcriptome and the translatome (2885 versus 2685), there is a strong drop in the number of flg22-activated genes that require TZF9 in the translatome (i.e. 322 + 108 versus 1122 + 51). These observations would imply that TZF9 is required to control the association of flg22-repressed/inducible mRNAs with actively translating ribosomes, which may be designated as ‘translational control’.

As for the transcriptome described above, we filtered the translatome data for genes mis-regulated in the tzf9 mutant (without flg22 stimulation) for possible candidates responsible for the attenuated defence response. This again revealed enrichment of stress/stimuli responsive genes in the tzf9 mutant in its uninduced state (Figures 4g and 5f, Table S4), thus showing some concordance between the transcriptome and translatome data. Interestingly, the most statistically significant GO terms among the genes downregulated in the tzf9 versus WT comparison included ‘translation’, ‘ribonucleoprotein complex biogenesis’ and ‘gene expression’ (Figure 5g). A closer examination of the ‘gene expression’ set of mis-expressed genes revealed mostly ribosome-related genes overlapping with the other two GO categories. This suggests that loss of TZF9 affects processes related to mRNA translation.

Widespread uncoupling of the transcriptome and translatome in tzf9 in its uninduced state

To compare the transcriptome and translatome datasets systematically, the consolidated DEGs (Figure 5a) were analysed separately (i.e. to dissect the effect of genotype or treatment). We first looked at genes affected by the tzf9 mutation (without any flg22 treatment) and plotted these as a heatmap or four-set flower Venn diagram (Figure 6a).
A logical assumption would be that most genes should display the same directional change in expression (i.e. up- or downregulated in both transcriptome and translatome) in the heatmap. However, such ‘homodirectional changes’ (marked in grey) are rare in the transcriptome dataset but not in the translatome dataset; yellow, DEGs observed only in the transcriptome dataset (‘Translatome only’); grey, ‘Antidirectional changes’ (i.e. up- or downregulation is consistent between transcriptome and translatome); magenta, antidirectional changes, i.e. DEGs up- or downregulated in the transcriptome are regulated in the opposite direction in the translatome).

The uncoupled genes in tzf9 show a translational bias compared with the WT upon flg22 elicitation

We know that PAMP elicitation also regulates gene expression through translational control (Xu et al., 2017). To understand the possible interplay between these two layers of translational control (i.e. via PAMP elicitation and additional translational control through TZF9), we compiled the transcriptome and translatome data of flg22-induced changes in Col-0 and tzf9 (Figure 6b, Tables S6 and S7). Unlike the comparison for the unelicited plants, the flg22-induced homodirectional change (coupled genes) is roughly similar (about 40%) in both Col-0 and tzf9 (Figure 6b). This rise from 2.29% (uninduced state) to about 40% (elicited state) coupled genes presumably reflects that PAMP-mediated translational control (Xu et al., 2017) is overriding the regulation executed by TZF9. The percentage of genes showing an ‘antidirectional’ pattern is negligible, so that the remaining 60% or so of the genes are either of the ‘transcriptome only’ (marked in blue) or ‘translatome only’ (marked in yellow) categories. However, when comparing the two genotypes, there is a shift in the distribution. The ‘translatome only’ category is almost twice as large in the tzf9 mutant as in the WT plants (47.55% versus 26.77%) (Figure 6b). This suggests that flg22-regulated gene expression in tzf9 is biased at the translational level compared with WT plants. Hence, at the biological level, TZF9 is involved in post-transcriptional regulation of gene expression, presumably in translational arrest since more mRNAs become associated with ribosomes in its absence.

The TZF9 protein physically associates with a stress granule protein and affects stress granule formation

One of the best known translational control mechanisms is translational stalling and arrest of mRNAs in stress granules (SGs) (Weber et al., 2008; Anderson and Kedersha, 2009). The poly(A)-binding proteins PAB2 and PAB8 are typically used as SG marker proteins for cell biology studies (Pomeranz et al., 2010a; Sorenson and Bailey-Serres, 2014). Accordingly, transiently expressed RFP-tagged PAB2/8 are cytoplasmic in unstarved cells but rapidly aggregate into mRNA–protein complexes upon a short heat treatment (42°C for 30 min) (Figure 7). To investigate if the translational arrest function of TZF9 might be linked to SGs, we co-expressed GFP-tagged TZF9 with RFP-tagged PABs and found that they co-localized in granular cytoplasmic structures. Deletion of the ankyrin repeat or the CCCH domains of TZF9 did not affect this co-localization (Figure S7). As expected, the number of SG structures increased upon heat treatment, and were mostly marked by simultaneous TZF9 and PAB2/8 signals. It is important to note here that in all these co-expression studies, the typically cytoplasmic PAB2/8 were already organized into a few SG-like structures before any stress (see Figure 7a,b). This suggests that TZF9 (overexpression) can promote the assembly of PABs into SGs.

We therefore tested if the two proteins can interact. After transient expression in protoplasts, co-immunoprecipitation (co-IP) was performed using GFP-trap beads to pull down GFP-tagged TZF9 and immunoblotting to detect co-eluting HA-tagged PABs. PAB2 could be co-immunoprecipitated but not PAB8. We also compared the partial or full phosphosite TZF9 mutants for the co-IP but there was no effect on the TZF9–PAB2 interaction (Figure 7c). To further

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validate the interaction, a bimolecular fluorescence complementation (BiFC) assay was employed where fluorescence signals were reconstituted by expressing YFP fragments fused to TZF9 and PAB2 (or PAB8), respectively (Figure 7d). Similar BiFC results were obtained with the TZF9 phosphosite mutant PS 1-14 (Figures 7d and S7), suggesting that the phosphosites are not essential for the interaction. However, unlike in the co-localization experiments, the YFP signals were cytoplasmic, with no (or only a few) SG structures seen. Heat stress, but not flg22 treatment, can (re)induce the formation of YFP-labelled cytoplasmic foci (Figure S8), suggesting that SG assembly of the TZF9-PAB complex is not generally perturbed. Taken together, TZF9 can interact with the SG protein, PAB2 (and also with PAB8, albeit with weaker affinity), and even promote nucleation into SGs of PABs prior to stress. It is likely that this interaction contributes to its function in translational arrest of associated mRNAs bound to PABs or to TZF9 directly.

DISCUSSION

Post-translational modifications (e.g. phosphorylation) can alter protein function. In this study, we have shown that, among the four flg22-responsive MAPKs (Bethke et al., 2012), MPK3 and MPK6 phosphorylate TZF9 and identified the major phosphosites (Figure 3). Paralogous TZFs have been reported to be phosphorylated, for example TZF7 by MPK3/6 (Feilner et al., 2005; Lassowskat et al., 2014; Lee et al., 2015; Latrasse et al., 2017) or TZF10 by the calcium-dependent protein kinase, CPK3 (Kanchiswamy et al., 2010). Even though other kinases may be active during flg22 elicitation, the MKK5–MPK3/6 module is likely the major phosphorylation cascade responsible for in vivo TZF9 phosphorylation since mutation of the corresponding phosphosites abrogated the flg22-induced phosphoshift (Figure 3g,h). Furthermore, the observed phosphoshift caused by mimicking MPK3/6 activation was comparable to that induced by flg22 elicitation (Figure S4).

Figure 7. Tandem zinc finger protein 9 (TZF9) interacts with stress granule (SG) proteins poly(A)-binding protein 2 or 8 (PAB2/8) and promotes stress-independent SG assembly.
(a) Red fluorescent protein (RFP)-tagged PAB2/8 were transiently expressed in protoplasts and the cytoplasmic localization at room temperature (RT) and SG formation after heat stress (42°C, 30 min) was visualized by confocal microscopy.
(b) Green fluorescent protein (GFP)-tagged TZF9 was co-expressed with RFP-tagged PABs and treated as above. Note the increase in SG structures after heat treatment but also the presence of PABs in cytoplasmic foci prior to stress.
(c) Co-immunoprecipitation of PAB2/8 with GFP-tagged TZF9 phosphosite variants. After transient expression in protoplasts, TZF9 was enriched from cell lysates using GFP-trap beads (IP) and the presence of co-precipitating PABs detected by anti-haemagglutinin (anti-HA) immunoblotting (co-IP). The lower panel is the compiled immunoblots of aliquots of the cell lysate (input) showing expression of the respective proteins.
(d) Bimolecular fluorescence complementation assays showing reconstitution of YFP signals after co-expressing the indicated protein fusions to N- or C-terminal halves of YFP fragments (nYFP or cYFP, respectively). PS 1-14 = phospho-null mutant of TZF9.
MAPK-targeted phosphosites are required for regulation of TZF9 turnover upon flg22 treatment. Besides being destabilised, some of the phospho-TZF9 was dephosphorylated 2 h after flg22 elicitation (Figure 3b), suggesting that there is tight in vivo control of the TZF9 phosphorylation state. Overall, TZF9 can join the list of plant enzymes (Li et al., 2012) and transcriptional regulators (Bethke et al., 2009; Meng et al., 2013; Pecher et al., 2014; Sheikh et al., 2016) whereby MAPKs regulate plant immunity through phospho-control of substrate stability. This MAPK-mediated phosphorylation suggests that TZF9 acts downstream of MAPKs. However, TZF9 was previously shown to be required for full MAPK activation (Maldonado-Bonilla et al., 2014) and therefore also acts upstream of MAPKs. This conundrum can probably be explained now by the indirect effects of the post-transcriptional control exhibited by TZF9 (see below), where signalling components essential for proper MAPK activation are compromised.

Localization of TZF9 suggests its mode of action to be within PB-like structures. Interestingly, numerous PB proteins have been reported to be phosphorylated by MAPKs, for example DCP1 (Xu and Chua, 2012) and PAT1 (Roux et al., 2015). Our previous phosphoproteomics-based screen also identified DCP1, Varicose, XRN4 and TZF7 as putative MKP3/6 substrates (Lassowskat et al., 2014). There is also increasing evidence linking PB functions to plant immunity. For instance, selective translational repression of viral (but not host) transcripts is associated with enhanced PB biogenesis (Meteignier et al., 2016) while SNC1 resistance gene-mediated autoimmunity is controlled by TAF15b, a mRNA export machinery component that localises to PBs (Dong et al., 2016). Thus, part of the MAPK-regulated plant immunity process may be modulated post-transcriptionally through PB functions.

Figure 8 summarises the main findings and implications of this work. Besides controlling (long-term) protein stability, phosphorylation affects the RNA-binding and PB aggregation properties of TZF9. While MKP3/6-targeted phosphorylation is required for the disintegration of TZF9-labelled PBs upon flg22 treatment, phosphorylation of PAT1 by MPK4 induces its localisation into PBs after PAMP treatment (Roux et al., 2015). These differential re-localisation patterns of PB components through distinct MAPKs highlights, besides separate functions, the dynamic nature of PB content, which could explain the discrepancy between reports on TZF localisation (Jang, 2016). The major phosphosites of TZF9 flank the CCCH domain (Figure 3a) and phosphorylation-induced conformational changes presumably led to reduced RNA-binding and rapid disassembly of the PB structures. This is in accordance with previous inhibitor-based studies showing the requirement for mRNA availability for localisation of TZF9 and TZF1 within cytoplasmic foci (Pomeranz et al., 2010b; Maldonado-Bonilla et al., 2014). Similarly, phosphorylation of tristetraprolin (TTP; the mammalian putative TZF orthologue) was thought to regulate binding of TTP to mRNAs (Hitti et al., 2006). The TTP protein is one of the best studied post-transcriptional regulators that binds AU-rich elements (AREs) and is phosphorylated by p38 MAPK and MK2 (a downstream target of p38 MAPK) (Chrestensen et al., 2004). However, subsequent work has shown that rather than attenuating RNA-binding, TTP phosphorylation prevents recruitment of the deadenylase machinery and thereby reduces mRNA decay (Clement et al., 2011).

Alternatively, phosphorylation may affect protein–protein interactions. Current analysis with the phospho-null mutants suggests phosphorylation is not essential for interaction with PBs but it might perhaps be relevant for other interactors. In this context, related TZFs have been shown to interact with numerous proteins, for example the Arabidopsis seed-specific TZF4-6 interacted with two stress-related proteins, MARD1 (mediator of ABA-regulated dormancy 1) and RD21A (responsive to dehydration 21A) within PBs and SGs (Bogamuwa and Jang, 2016). Similarly, the cotton GhZFP1, which is a homolog of Arabidopsis TZF4-5, interacted with PR5 (pathogenesis-related protein 5) and RD21A (Guo et al., 2009). Notably, the interaction required the CCCH domain and occurred in the cytoplasm and nucleus, possibly hinting at distinct compartment-specific functions. Although localised in PBs, Arabidopsis TZFs do not directly interact with any PB marker proteins in yeast two-hybrid analysis (Bogamuwa and Jang, 2016). While it is part of the mRNA decapping complex within PBs, mammalian TTP also co-immunoprecipitated and co-localised with non-PB proteins (Brooks and Blackshear, 2013). This is determined via distinct interacting domains of TTP, for instance its carboxyl terminus for interaction with the cytoplasmic hCIN85 (human Cbl-interacting protein85) and its TZF domain with the nuclear PABP8 (polyadenylate binding protein8) (Kedar et al., 2010; Su et al., 2012). Here, we showed that TZF9 can also directly interact with cytoplasmically localized PABs and stimulate their nucleation into SG-like structures. The current designation of the TZF9-labelled cytoplasmic foci as PBs or SGs (Figure 8) may hence be inaccurate and, until more is known, these structures should probably be referred to as RNA granules.

The original central dogma of molecular biology proposes a ‘simple’ flow of genetic information decoding from DNA to mRNA to protein. However, due to post-transcriptional control, there is often ‘uncoupling’ between transcription and translation. On the basis of its PB co-localisation and analogy to TTP, an initial hypothesis was that TZF9 controls RNA decay. As reported here, the transcriptome–translatome discordance is particularly strong in the tzf9 mutant; with a shift towards ‘translatome only’ changes in the tzf9 mutant, we now propose that TZF9 associates with certain mRNAs and determines their
translatability’ (Figure 8). This is congruent with the notion that in the absence of TZF9, certain RNAs are no longer sequestered into RNA granules and are free to associate with translating ribosomes (Figure 8). Our observation of rapid flg22-induced disruption of TZF9-labelled foci could be evidence for such a release from translational arrest upon PAMP treatment. Thus, TZF9 may function analogously to the oligouridylate-binding protein 1 (UBP1), an RNA-binding protein that binds U-rich 3' untranslated regions (UTRs) of subpopulations of mRNAs and determines relief from translation repression during hypoxia stress (Sorenson and Bailey-Serres, 2014; Chantarachot and Bailey-Serres, 2018). A caveat in our experimental set-up and interpretation is that the rapid disruption of TZF9-labelled granules (within minutes) may not necessarily reflect the transcriptome–translatome results at 1 h of flg22 treatment. Thus, it will be crucial in future analysis to check the long-term dynamics of TZF9-marked RNA granules in possibly different phosphorylation states of TZF9 as well as effects from altered TZF9 stability.

Recently, a second layer of control of translational efficiency that is triggered by PAMPs was described (Xu et al., 2017). This PAMP-triggered translational control is mediated by binding of PABs to a purine-rich element found in the 5' UTRs of some PAMP-responsive genes. Since TZF9 can interact directly with PAB2 and promote its assembly into SGs, two crucial questions arise here: (i) Does the TZF9–PAB2 complex determine the ‘translational bias’ observed with the tzf9 mutant? This could occur through classical mRNA sequestration/translational arrest ascribed to SGs. Alternatively, TZF9 interacting with PABs may compete and interfere with optimised protein translation in circular closed-loops mRNA structures (bottom), where PABs link 3' poly(A) tails to the 5' mRNA cap structures through the translational elongation initiation factor, elf4E, and the adaptor protein, elf4G. Such circular mRNA loop topology promotes ribosome recycling and multiple ‘rounds’ of protein translation. Not included in the model is the role of PABs in pathogen-associated molecular pattern-mediated translational control by binding a 5’ untranslatable region purine-rich motif (Xu et al., 2017). DCP, decapping complex; XRN, ribonuclease for 5'–3' degradation; CCR4-NOT, deadenylate and 3'-5' degradation enzymes.
analysis on the dataset of mis-expressed genes in the translatome of the unelicited tzf9 mutant, an AG-rich element similar to the 5’ UTR purine-rich motif described for PAMP-mediated translational control (Xu et al., 2017) as well as a second pyrimidine-rich element were detected. Notably, these were enriched in both the 5’ and 3’ UTRs (Figure S9). The pyrimidine-rich 3’ UTR element has fewer cytosines and may be considered as U-rich (i.e. reminiscent of the U-rich/ARE-like sequences) and therefore bind directly to TZF9. Here, one needs to take into account two limitations: (i) TZF9 may bind folded RNA structures, thus precluding the identification of short consensus linear cis-elements with the current assays. (ii) The SELEX analysis recovered only a small number of binding sequences. Bearing these points in mind, the consensus TZF9-associated U-rich or ‘UAAA’ motifs (Figure 1d) can be matched to the U-rich/pyrimidine-rich element or purine-rich sequences, respectively. While it is possible that TZF9 may bind the same purine-rich elements as the PABs involved in PAMP-triggered translational reprogramming, it is also possible that our MEME analysis revealed indirectly the targets of PABs that were in complex with TZF9. In fact, this may be the explanation for the unexpectedly high number of flg22-downregulated genes (i.e. 1460) in the ‘translatome only’ category of DEGs in the tzf9 mutant (Figure 6b), which may include many indirect targets. Otherwise, according to our premise of a release from translation arrest, one would have expected more flg22-upregulated DEGs when TZF9 is absent. In any case, a more appropriate strategy for unravelling the true sequence element(s) targeted by TZF9 in vivo would be to use individual-nucleotide resolution UV crosslinking and immunoprecipitation sequencing (iCLIP-seq) (Stork and Zheng, 2016) to identify natively bound RNAs. Such analysis in the future will improve differences between the mis-expressed genes in the tzf9 mutant and the mRNAs bound directly to TZF9.

In conclusion, TZF9 appears to be involved in translational arrest/repression in unstimulated plants; it may be released through the MAPK-mediated control described above and/or through the recently described PAMP-mediated translational control (Xu et al., 2017). Both of these pathways may in fact be connected since they share PABs as common elements. Since PABs are SG components, SG-like RNA granules may be the sites for control of translational arrest during pathogen stress.

**EXPERIMENTAL PROCEDURES**

**Plant growth conditions and genotypes**

All the plants were grown under short-day conditions (8 h light, 22°C and 16 h dark, 20°C). *Arabidopsis thaliana* (ecotype Col-0) wild-type (WT) and tzf9 knockout plants (NASC ID: N510842) aged about 6 weeks were used for the transcriptome analysis. For the translatomics experiment, *A. thaliana* expressing the FLAG-tagged ribosomal gene L-18 (RPL18) under the control of the CaMV 35S promoter was used (Mustroph et al., 2009). This p35S::RPL18 transgenic line was crossed with the tzf9 knockout line and homozygous lines (p35S::RPL18/tzf9) were selected in the F2 generation. The TZF9-overexpressing transgenic Arabidopsis line (p35S::TZF9-HA-YFP) was generated by the floral dip method (Logemann et al., 2006) using *Agrobacterium tumefaciens* harbouring TZF9 cloned in the pEarley101 vector (Earley et al., 2006). After selection, the T3 generation was used for the experiments (see also Data S1).

**Transcriptomics and translatomics**

Arabidopsis Col-0 (WT), tzf9, p35S::RPL18 and p35S::RPL18/tzf9 lines were infiltrated with flg22 (1 µm) and harvested after 1 h. Multiple leaves were collected per treatment and triplicate individual pools of independently collected leaves were processed for the following steps. For transcriptomics, total RNA was extracted from the homogenised leaves of Col-0 and tzf9 using TRIzol® reagent. For translatomics, polysomes were extracted from the homogenised leaves of p35S::RPL18 and p35S::RPL18/tzf9 plants, immunoprecipitated with anti-FLAG M2 agarose resins (Sigma, https://www.sigmaaldrich.com/) and the RNA isolated as described in Mustroph et al. (2009). For transcriptomics 100 ng of total RNA and for translatomics 150 ng of mRNAs were used for cDNA synthesis (GeneChip™ WT PLUS Reagent Kit; Thermo Scientific, https://www.thermofisher.com/). Complementary DNAs were subsequently labelled with biotin and microarray hybridization was performed on an Affymetrix® Arabidopsis Gene 1.1 ST Array Strip (cat. no. 901793, https://www.affymetrix.com/) according to the instructions in GeneAlts™ Hybridization, Wash, and Stain Kit for WT Array Strips the manual (cat. no. 901667, https://www.thermofisher.com/). Hybridization data were pre-processed by the R package XPS. Raw data were normalized by robust multi-array average expression measure, which includes probe-set summarisation and quantile normalisation. The dataset was filtered for unexpressed features by detection above background calls. Genes were retained if all signals of at least one replicate group were detected in either of the two genotypes. Linear models were fitted for each feature using LIMMA (Ritchie et al., 2015) and P-values were adjusted by the false discovery rate procedure of Benjamini–Hochberg (Benjamini and Hochberg, 1995).

**ACCESSION NUMBERS**

The following genes were described in this manuscript: At5g58620 (TZF9), At3g45640 (MPK3), At2g43790 (MPK6), At4g34110 (PAB2), At1g49760 (PAB8), At1g09370 (DCP1). The microarray raw data are available on the ArrayExpress website under http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6918.

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AUTHOR CONTRIBUTION

NT and LE-L performed most of the presented experiments. MB provided the data concerning PAB-TZF interactions. MBr performed the SELEX experiments. LDM-B provided the pioneering pilot experiments that served as basis for this work. BA handled the bioinformatics analysis of the microarray. WH performed the phosphosite identification. GH provided the EM imaging. DS and JL supervised the project. JL coordinated the writing together with all authors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Tandem zinc finger protein 9 binds to pentaprobe-2 in a RNA electrophoretic mobility shift assay.

Figure S2. Tandem zinc finger protein 9 binds short RNA sequences in systematic evolution of ligands by exponential enrichment.

Figure S3. The MS profile shows phosphorylation at respective residues.

Figure S4. Phospho-mobility shift of tandem zinc finger protein 9 is due to phosphorylation of mitogen-activated protein kinases MPK2/6.

Figure S5. Localisation of changes in wild-type tandem zinc finger protein 9 upon flagellin treatment.

Figure S6. The real-time quantitative PCR validation of selected differentially expressed genes from the microarray.

Figure S7. Effect of ankyrin repeat or CCCH domain deletion on co-localization of poly(A)-binding protein and tandem zinc finger protein 9.

Figure S8. Bimolecular fluorescence complementation analysis of interaction between tandem zinc finger protein 9 (and a phosphosite variant) and poly(A)-binding proteins 2 and 8.

Figure S9. MEME analysis of common motifs in the 5’ or 3’ untranslated regions of the mis-expressed genes in the translatome of the tzf9 mutant compared with the wild type (without pathogen-associated molecular pattern elicitation).

Table S1. List of up- and downregulated genes in the transcriptome.

Table S2. List of up- and downregulated genes in the translatome.

Table S3. Functional enrichment of genes up- and downregulated in tzf9 in the uninduced state (transcriptome).

Table S4. Functional enrichment of genes up- and downregulated in tzf9 in the uninduced state (translatome).

Table S5. List of deregulated coupled and uncoupled genes in tzf9 in the uninduced state.

Table S6. List of flagellin-induced or repressed-coupled and uncoupled genes in the wild type.

Table S7. List of flagellin-induced or repressed coupled and uncoupled genes in tzf9.

Table S8. List of primers.

Data S1. Supplementary methods.

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