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FORMATION OF *ARABIDOPSIS* POLY(A)-SPECIFIC RIBONUCLEASE ASSOCIATED PROCESSING BODIES IN RESPONSE TO PATHOGENIC INFECTION

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Abstract

In eukaryotes, the fate of messenger RNA (mRNA) is tightly regulated. Identifying the role of specific regulatory proteins involved in post-transcriptional gene regulation is highly intriguing. Specific cytoplasmic foci known as the processing bodies (P-bodies) play a critical role, as it harbours numerous protein complexes which are involved in degradation and translational arrest of mRNA. Essentially, the protein components present in P-bodies are primarily involved in mRNA decapping, deadenylation and decay processes. Among several ribonucleases involved in modification of the mRNA, poly(A)-specific ribonuclease (PARN) specifically facilitates the deadenylation of mRNA. Deadenylation is considered to be one of the critical events which ensure the fidelity of mRNA. The physiological role of PARN during biotic stress is not yet fully revealed. Here we report that under pathogen challenged conditions, PARN relocalizes to the cytoplasm and the number of P-bodies induced by the PARN is significantly reduced. This identifies that the molecular functioning of PARN is affected when plants are exposed to pathogens and may be well involved in contributing for plant defense.

Key words: messenger RNA, gene regulation, processing bodies, deadenylation, poly(A)-specific ribonuclease.

Introduction

Throughout the entire process of transcription and post-transcription, regulatory mechanisms ensures complete fidelity by restricting any faulty messenger RNA (mRNA) to arrive into the translational event. Even, the physiological mRNAs needs to be safely disposed off the cellular events in order to maintain homeostasis. Thus, the decay of mRNA is critical and determines the steady-state concentration of cellular transcripts. Essentially, there are four known mRNA degradation pathways in Fungi and Metazoans: (1) nonsense-mediated decay (Chen and Shyu, 2003; Lejeune *et al.,* 2003; Mitchell and Tollervey, 2003; Panigrahi and Satapathy, 2020a); (2) deadenylation-dependent decapping resulting in 5'-3' decay (Mitchell and Tollervey, 2000; Tucker and Parker, 2000; Butler, 2002); (3) deadenylation-mediated 3'-5' decay (Mitchell and Tollervey, 2000; Tucker and Parker, 2000; Butler, 2002); (4) nonstop decay (Frischmeyer *et al.,* 2002; van Hoof *et al.,* 2002). Strikingly, each of these mRNA degradation pathways requires the deadenylation event to occur. Essentially, deadenylation process serves as a rate-determing step during the mRNA degradation and regulates the overall steady-state level of mRNA (Sergei *et al.,* 2004; Cao and Parker, 2001). Primarily, deadenylation is tightly regulated by several protein factors which ultimately recruit deadenylases, like Poly(A) ribonuclease (Winzen *et al.,* 1999; Vasudevan and Peltz, 2001). Poly(A) ribonuclease (PARN) harbours four conserved acidic amino acid residues and it belongs to the RNase D family of nucleases (Wilusz *et al.,* 2001). PARN specifically targets the poly(A) tail, though its activity is highly regulated by the mRNA 5'm7GpppG cap (Dehlin *et al.,* 2000; Gao *et al.,* 2000; Martinez *et al.,* 2001). In plants, the role of PARN is not clearly deciphered. Several embryo-specific transcripts are regulated by the PARN as confirmed in the mutant plants (Sergei *et al.,* 2004). It was then inferred that PARN role in the deadenylation of specific transcripts during the developmental stage is critical. Conspicuously, the absence of PARN in the *Schizosaccharomyces pombe* did not displayed any adverse effect as observed in case of plants (Sergei *et al.,* 2004). Thus, it reflects the non-universal role of PARN across eukaryotes.

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Essentially, the mRNA decay pathways across eukaryotes play a significant role in removing the non-physiological mRNA from the cellular environment (Houseley and Tollervey, 2009; Christie *et al.,* 2011). Moreover in eukaryotes, the decay of mRNAs occurs in specific cytoplasmic foci, known as processing bodies (Bashkirov *et al.,* 1997). Both the decapping and deadenylation protein factors are recruited on the mRNA which is destined for degradation (Panigrahi and Satapathy, 2020b). Interestingly, the processing bodies (P-bodies) harbour 5' and 3' ribonucleases (Sheth and Parker, 2003; Eystathioy *et al.,* 2003). The components and composition of P-bodies is highly conserved across eukaryotes (Parker and Sheth, 2007; Bonila, 2014). In the P-bodies, translationally repressed mRNAs aggregate and eventually gets degraded. The decapping and deadenylation of mRNA is primarily catalyzed in the P-bodies. The deadenylation of mRNA is mostly facilitated by the deadenylizing proteins, PARN and catabolite repressor 4 (CCR4) - CCR4 associated factor 1 (CAF1) complex (Moreno *et al.,* 2013). PARN and CCR4-CAF1 complex co-localize with the decapping protein 1 (DCP1) in the P-bodies (Moreno *et al.,* 2013).

Primarily, the objective of this study was to see any changes in the localization of PARN, when the plant is exposed to pathogen. Strikingly, the PARN associated P-bodies count significantly declined post pathogen infection. This previously unknown phenomenon may hint out towards the involvement of PARN during biotic stress. Further, it is speculated that the interconnection between PARN and P-bodies may be well involved in regulating the expression of specific transcripts which would be involved during plant defense response.

Materials and Methods

Plant materials and growth conditions

Nicotiana benthamiana plants were grown in soil, essentially for transient expression of proteins. Localization and Western blotting assays were done using the leaves of *Nicotiana benthamiana*. The plants were grown at 22°C under long day (12/12 h light/dark) conditions with 70-80% relative humidity.

Plasmids

Full-length cDNA of PARN was obtained by RT-PCR using Col-0 RNA. The purified PCR products were cloned into the entry vector and further sequencing was done for confirmation. Wild type (WT) coding sequences of PARN were cloned into expression vectors for microscopy and immunoblotting assays. The primer sequences 5' *ATGCGCCGGCACAAGCGAT* 3' (forward primer) and 5' *ATTACTCGTAGCAGTTTCGAC* 3' (reverse primer) were used for amplifying the open reading frame using cDNA as template for polymerase chain reaction (PCR).

Pathogen infection assay

Pseudomonas syringae strains were cultured for overnight at 28°C in the King's B medium along with spectinomycin and rifampicin as antibiotics. Bacteria were harvested by centrifugation at 3000 rpm for 15 minutes and washed thrice with double distilled H_2O. The pellet was resuspended with 10 mM $MgCl_2$ and adjusted to the density of $OD_{600}=5\times10^{-4}$. Leaves of four-week-old *Nicotiana benthamiana* plants were hand-infiltrated with bacterial suspension using a needleless syringe. At different time points (0, 15, 30, 60, 90, 120, 180, 240 minutes post infection) the leave samples were collected for localization and immunoblotting assays.

Agro-infiltration and confocal laser scanning microscopy (CLSM)

The binary clones corresponding to PARN were transformed into *Agrobacterium tumefaciens* strain and were agro-infiltrated into *Nicotiana benthamiana* leaves. Two days post-agroinfiltration, the injected leaves were analyzed under CLSM. For the CLSM assay, the GFP and bimolecular fluorescence complementation (BiFC) constructs were used.

Protein extraction and Western blotting

Total proteins were extracted from roughly 0.1g leaf of the *Nicotiana benthamiana* plants, injected with the *Agrobacterium tumefaciens* strain harbouring the protein constructs, PARN-HA. The plants were grown at 22°C and under long day (12/12 h light/dark) conditions. The protein expressing leaves were harvested at different time points (0, 15, 30, 60, 90, 120, 180, 240 minutes post infection). The extraction buffer composed of 20 mM of Tris-Cl (pH 8.0), 100 mM of NaCl, 1 mM of EDTA, 1 mM of PMSF, and 1X proteinase inhibitor. The homogenate obtained after centrifugation was resuspended in SDS sample buffer and loaded on SDS-polyacrylamide gels for subsequent Western blot analysis. Monoclonal α-HA, α-Actin antibodies were used for Western blotting.

Statistical analysis

The number of P-bodies observed in a specific cell was calculated. The total area of the cell and total number of P-bodies occurring in the cell were recorded. Experiments were done in triplicates to validate the results.

Results and Discussion

Arabidopsis PARN is a highly conserved protein across various plant species

The *Arabidopsis thaliana* gene, At1g55870 (*AtPARN*) encode protein with high sequence similarity to metazoan PARN (BLAST E values of $6 \times e^{-39}$ upon using human PARN as a query, fig. 1). The *AtPARN* open reading frame (ORF) contains all of the four highly conserved acidic residues shown earlier to be essential for catalysis in the RNase D family nucleases, including human PARN (Ren *et al*., 2002). The protein sequence of the PARN is highly conserved across various plant species (Fig. 1). The protein sequence of the *Arabidopsis* PARN shares maximum number of fully conserved residues relative to other plant species. Very few amino acid residues correspond to non-conserved segment. The extreme conservancy of the *Arabidopsis* PARN across different plant species suggests that they all are equipped with this vital protein factor which is canonically involved in the mRNA homeostasis. PARN play a significant role in maintaining the RNA turnover and thus a number of plants harbour them to effectively act against the stress conditions.

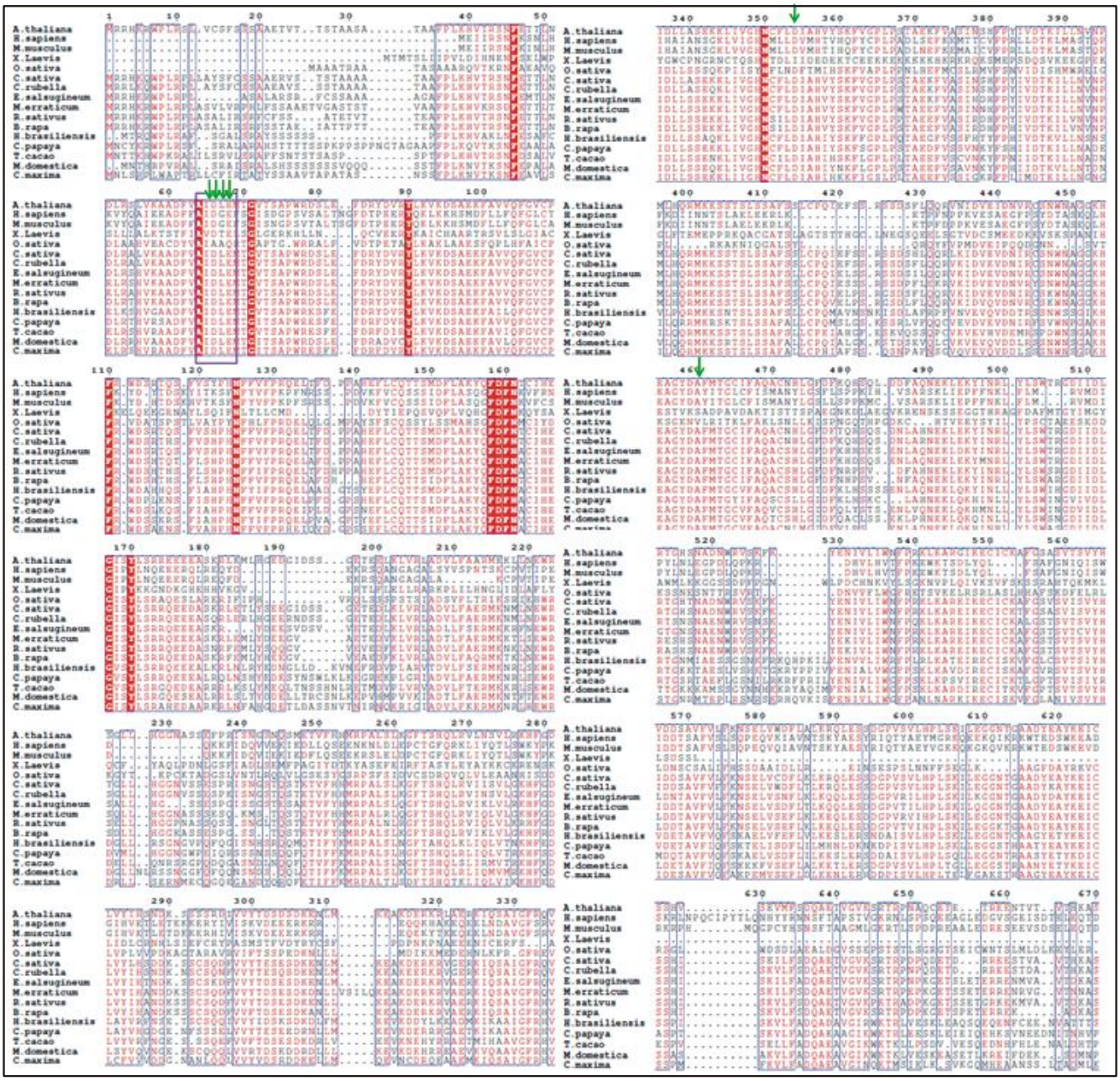

Fig 1: Protein sequence alignment of the PARN of *Arabidopsis thaliana*. Residues that are 100% conserved are in red boxes. Similarity >70% is red. Figure was generated with Multalign and ESPript (Robert and Gouet, 2014). The amino acid residues indicated with arrow marks primarily represents the highly conserved acidic residues essential for catalysis.

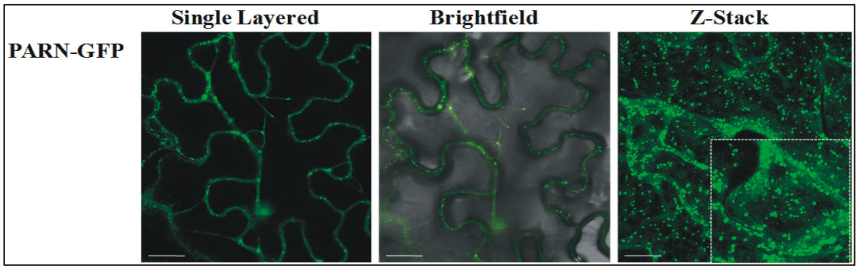

Fig. 2: Localization of PARN-GFP. The *Arabidopsis* PARN localizes predominantly in the cytoplasm and the P-bodies. The proteins were transiently expressed in the *Nicotiana benthamiana*. (Scale bar: 10µm).

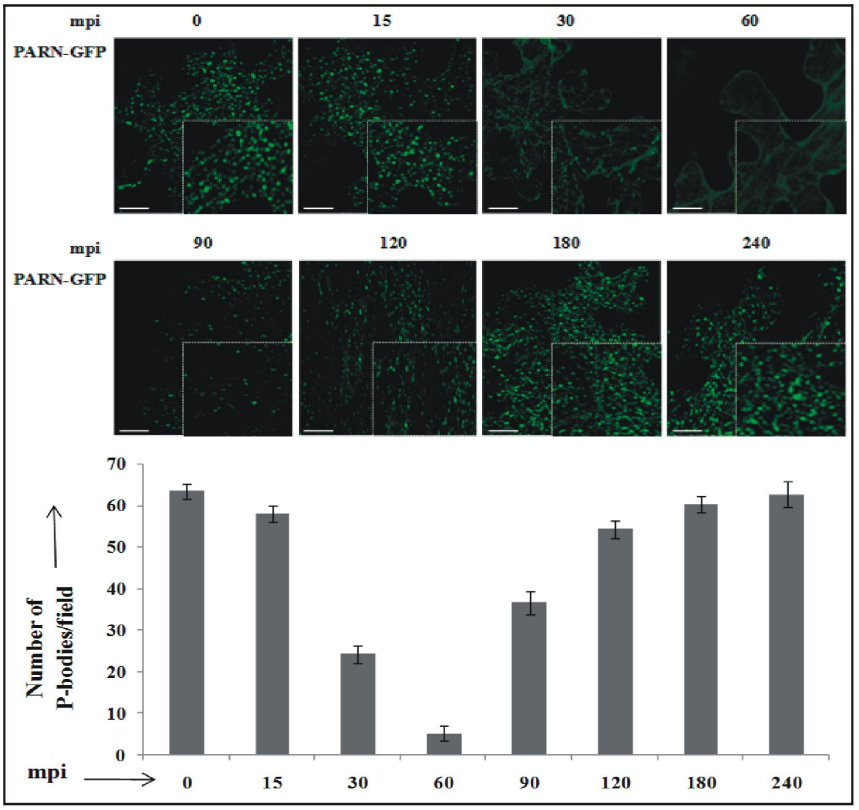

Fig. 3: Dynamics of localization of PARN-GFP upon pathogen infection. The *Arabidopsis* PARN differentially localizes under pathogen challenged conditions. The proteins were transiently expressed in the *Nicotiana benthamiana*. (Scale bar: 10µm). Localization of the *Arabidopsis* PARN was observed at different time points including 0, 15, 30, 60, 90, 120, 180, 240 minutes post infection (mpi). Number of PARN associated P-bodies were significantly declined at 30 and 60 mpi.

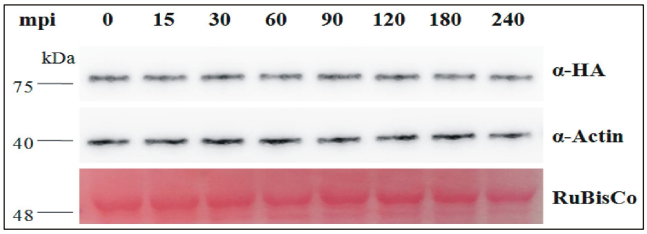

Fig. 4: The protein level of the *Arabidopsis* PARN remains unaffected at different time points post pathogen infection. The Western blots were generated using α-HA and α-Actin antibodies. Protein loading is shown by Ponceau S staining for RuBisCo.

Dynamics of PARN-induced P-bodies in *Nicotiana benthamiana*

Essentially, PARN being involved in the deadenylation process of mRNA and one of the primary components of the deadenylation complex, PARN-GFP localizes to the P-bodies when transiently expressed in *Nicotiana benthamiana* (Fig. 2). To reveal the potential link between PARN-induced P-bodies and plant immunity, the leaves of *Nicotiana benthamiana* were infected by *Pseudomonas syringae*. The dynamics of PARN-induced P-bodies upon pathogen infection leading to the elicitation of pathogen triggered immunity was explored. Interestingly, it was revealed that there was a significant reduction of PARN-associated P-bodies at 30 and 60 minutes post infection (mpi; Fig. 3). Strikingly, the PARN-associated P-bodies again re-appeared at 90 mpi (Fig. 3). Together, the results indicate that the physiological function of PARN is not only limited to its involvement in the deadenylation of mRNA but also may be associated in the plant defense response.

The protein level of PARN is unaffected upon pathogen infection

Apparently, the decline of PARN-associated P-bodies (Fig. 3) was not due to the reduced protein expression of PARN protein as evident from the immunoblotting assay, suggesting that the reduction of PARN-associated P-bodies upon pathogen infection may be due to disassembly of P-body leading to the differential localization of PARN (Fig. 4). Immunoblotting assays clearly indicate that the level of PARN protein did not get affected at the 30 and 60 mpi (Fig. 4). Since, there was a reduction in the number of P-bodies at 30 and 60 mpi, it was expected that this could be the outcome of decrease in the PARN protein expression, but this is not the case. There may be several possibilities that the PARN may be well involved in the regulation of plant defense mechanism, which would eventually require the PARN to relocalize from the P-bodies into the cytoplasm, so as to participate in defense related cascades. The results indicate that under

pathogenic stress, the PARN may differentially localize in the cell to get involved in escalating the plant immune response.

Conclusion

Dynamic transcriptome reprogramming occurs when a plant is challenged by an incoming pathogen, leading to a robust defense response. The multi-levelled post transcriptional events significantly control the plant immune response. The turnover of mRNA which predominantly occurs in the P-bodies is tightly regulated, mediated by several protein factors. PARN is a key component of the mRNA deadenylation complex. The differential expression of PARN in terms of its physical localization when plant is exposed to pathogen reveals its non-canonical role. The prima facie evidence that the PARN relocalizes from the P-bodies into the cytoplasm points out for a possibility of its involvement in the plant defense response. There is equal possibility that the PARN may specifically regulate key immune related genes which would otherwise not express when the plants are unchallenged by any pathogen. As soon as the plant gets exposed to any invading pathogen, the PARN may allow specific mRNA transcripts to translate, which may trigger downstream defense related responses. The loss-of-function of PARN in plants severely affects the plant viability (Sergei *et al*., 2004). Thus, the *Arabidopsis* PARN which is highly redundant among several plant species and metazoans is involved in regulating the translational efficacy and fine tunes the expression of specific transcripts. Thus, our study lays down a previously unknown physiological role of *Arabidopsis* PARN and future studies may well reveal the unravelled connecting link between mRNA deadenylation process and pathogen triggered immunity.

Author contribution statement

Kunja Bihari Satapathy and Gagan Kumar Panigrahi conceived the idea. Gagan Kumar Panigrahi performed the experiments. Kunja Bihari Satapathy and Gagan Kumar Panigrahi analyzed the results. Gagan Kumar Panigrahi wrote the manuscript and Kunja Bihari Satapathy made necessary corrections in the draft.

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Conflict of interest

The authors declare that they have no conflict of interest.

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