Article

Arabidopsis PII proteins form characteristic foci in chloroplasts indicating novel properties in protein interaction and degradation

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Abstract: The PII protein is an evolutionary highly conserved regulatory protein from bacteria to higher plants. In bacteria it modulates the activity of several enzymes, transporters and regulatory factors by interacting with them and thereby regulating important metabolic hubs like carbon/nitrogen homeostasis. More than two decades ago the PII protein was characterized for the first time in plants, but its physiological role is still not sufficiently resolved. To gain more insights into the function of this protein, we investigated the interaction behaviour of AtPII with candidate proteins by BiFC and FRET/FLIM *in planta* and with GFP/RFP traps *in vitro*. In the course of these studies we found that AtPII interacts in chloroplasts with itself as well as with known interactors like NAGK in dot-like aggregates, which we named PII foci. In these novel protein aggregates AtPII interacts also with yet unknown partners, which are known to be involved in plastidic protein degradation. Further studies revealed that the C-terminal part of AtPII is crucial for the formation of PII foci. Altogether, the presented results indicate a novel mode of interaction for PII proteins with other proteins in plants, which may be a new starting point for the elucidation of physiological functions of PII proteins in plants.

Keywords: plant PII protein; protein-protein interaction; PII foci; BiFC; FRET/FLIM; plastidic protein degradation; cpUPR

1. Introduction

PII signalling proteins are ubiquitously distributed in all prokaryotes and have been conserved in the evolution of the "green lineage". Therefore, PII proteins are found in all plants, from cryptogams to angiosperms, where they are almost exclusively localized in the plastids. In prokaryotes, PII proteins are widely distributed in bacteria and as well in many archaea [1, 2]. The trimeric proteins have a highly conserved structure and regulate their targets through tight protein-protein interactions, mainly mediated through a flexible, solvent loop structure of about 18 amino acids, the so-called T-loop. The T-loop can adopt various conformations, depending on the effector molecules ATP, ADP or Mg-ATP plus 2-oxoglutarate, bound in the three effector binding sites, which are located in the clefts between the subunits. Thereby the levels of the carbon/nitrogen status-reporter 2-oxoglutarate as well as the energy state, sensed by the ATP to ADP ratio, are integrated by PII and allow PII to control a multitude of cellular functions, mainly related to nitrogen assimilation but also to central carbon flux and other core features of metabolism such as NAD-synthesis [3, 4].

In cyanobacteria, the phylogenetic ancestors of chloroplasts of the plant kingdom through endosymbiosis, PII signalling has been investigated in detail [5]. The signalling

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principles are highly conserved as compared to heterotrophic bacteria, however, cyanobacteria appear to have evolved some specific PII regulatory targets. The controlling enzyme of the arginine pathway, N-acetyl-L-glutamate kinase (NAGK), appears to be a dominant target in these organisms, although recent studies revealed PII-NAGK interaction also in non-photosynthetic bacteria [4]. Moreover, in cyanobacteria PII controls the flux of newly fixed carbon by controlling a protein that acts as inhibitor of phosphoglycerate mutase (PGAM) [6].

In 1998 the first plant PII protein could be identified and characterized from *Arabidopsis*. It was surprising that the amino acid sequence revealed an identity of 50% or more to homologous protein sequences from *E. coli* or cyanobacteria [7]. Due to the degree of conservation of PII proteins from bacteria to plants, similar regulatory mechanisms as described above were expected to be mediated by these proteins in plants. Transcription of the corresponding gene *AtPII* revealed to be under control of carbohydrates and nitrogen, indicating a regulatory role of *At*PII in C/N homeostasis. In further studies, it was reported that plant PII proteins also bind the NAGK enzyme like in bacteria [8, 9]. This interaction had been shown to activate NAGK enzyme activity and to be strictly regulated by different metabolites like ATP, 2-oxoglutarate or glutamine [10-13]. The glutamine dependence was identified in the green alga *Chlamydomonas reinhardtii* [13] and could be resolved to be due to a glutamine-binding C-terminal extension of plant PII proteins. Strikingly, this extension is modified in *Arabidopsis thaliana* in such a way, that *At*PII binds NAGK in a glutamine independent manner. The evolution of glutamine sensing by plant PII proteins was recently reviewed in [14].

Additionally, also the interaction of Biotin Carboxyl Carrier Proteins (BCCPs) could be shown for plant PII proteins [12] indicating a regulatory role in fatty acid biosynthesis., which was later found to occur also in bacteria [15, 16].

Nevertheless, major physiological phenomena in plants under control of PII proteins are still awaiting to be unravelled. Arabidopsis plants overexpressing *AtPII* showed enhanced anthocyanin accumulation by glutamine application [7]. This led to the hypothesis of PII in plants as a hub in C/N balance, which was supported by the finding of direct glutamine binding of these proteins [13]. In contrast, the phenotype of PII overexpressing plants was unexpectedly moderate. Homologous overexpression of PII in *Lotus japonicus* led to deregulation of polyamine content and nodule number under high nitrogen supply [17] as well as to reduced water loss due to altered stomatal opening [18], but no drastic alteration of plant phenotype was reported. Also knock-out and knock-down *AtPII* plants were phenotypically rather slightly affected [19]. The utilization of *AtPII* mutants revealed reduced contents of arginine biosynthesis metabolites under certain nitrogen supply conditions [20] and increased uptake of nitrite in chloroplasts [21].

Altogether these findings led to the conclusion that PII is not crucial for plant nitrogen sensing [22]. Instead, it turned out that several proteins like the TOR (Target of Rapamycin) kinase, the GCN2 protein kinase, GLRs (Glutamate receptor-like proteins) or several other candidates may have taken over the task of nitrogen sensing in plants (for summaries see [10, 22]. Among them the TOR signalling pathway appears to play a central integrative function in this respect [23]. It was speculated that PII is just responsible for the upregulation of arginine biosynthesis under high glutamine supply [22]. In this case the question about the function of PII proteins in *Arabidopsis* and other Brassicaceae would remain, as these proteins had lost their glutamine binding moiety [13].

Moreover, the variety of PII interaction partners in bacteria and plants, the different metabolic pathways regulated by PII in bacteria, together with the degree of PII conservation in the course of evolution indicate a more pronounced role for this protein also in plants, which awaits to be unravelled. When we started our studies on the interaction partners and functions of the PII protein in *Arabidopsis* we came across a specific aggregation behaviour of this protein in plastids, which seems to be mediated by the C-terminal part of the protein. Further investigations of this phenomenon led to the discovery of novel interaction partners of *At*PII within this suborganellar structures, which indicated a central role of plant PII proteins in a novel context.

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2. Results

2.1. AtPII tagged with fluorescent proteins forms dot-like aggregates in chloroplasts

First attempts to analyse the subcellular localization of *At*PII revealed that GFP tagged versions of this protein under control of the 35S CaMV-promotor (*p35S CaMV::AtPII*$_{cDNA}$*-GFP*) were found in chloroplasts of transiently transfected tobacco cells. Most interestingly, they appeared there as roundly shaped dot-like or focal aggregates of varying size (Figure 1a). To exclude the possibility that these aggregates evolved due to overexpression phenomena, a GFP fusion of the genomic PII sequence driven by its endogenous promotor (*pAtPII::AtPII*$_{genomic}$*-GFP*) was introduced into tobacco cells. The expression of this construct also showed the plastidial aggregation (Figure 1b). Furthermore, the same construct was used for stable transformation into *Arabidopsis thaliana* Col-0 plants. Also, in the cells of these transformed plants, *At*PII-GFP aggregated in foci in chloroplasts as observed before in tobacco cells (Figure 1c), which shows that this phenomenon was not caused by heterologous overexpression. Because we observed this focal aggregation of PII proteins regularly, as it can be seen in the course of this report, we coined this phenomenon as PII foci.

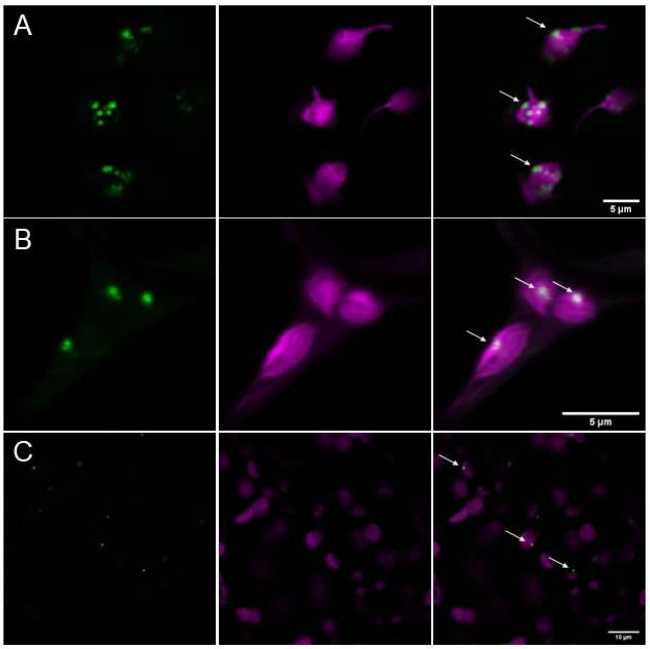

Figure 1. *At*PII aggregates in focal structures in chloroplasts. A) *At*PII-GFP (green) under the control of *p35S* (*p35S CaMV::AtPII*$_{cDNA}$-GFP) co-expressed with mCherry-tagged transit peptide of tobacco Rubisco (CD3-999 pt-rk [24]; magenta) localizes to plastids in transiently transformed *N. benthamiana* 2 days after infiltration. B) Genomic *At*PII-GFP (green) expressed under the control of the endogenous PII promoter (*pAtPII::AtPII*$_{genomic}$-GFP) and co-expressed with mCherry-tagged transit peptide of tobacco Rubisco (CD3-999 pt-rk; magenta) localizes to plastids in transiently transformed *N. benthamiana* 2 days after infiltration. C) Genomic *At*PII-GFP (green) under the control of endogenous *pAtPII* (*pAtPII::AtPII*$_{genomic}$-GFP) localizes to plastids (magenta) in stably transformed *A. thaliana*. In each row first the GFP fluorescence, second the mCherry fluorescence, and last the merge of both pictures is shown. White arrows mark exemplarily *At*PII aggregates.

The specific subcellular localization pattern of *At*PII raised the question if formation of PII foci is a general phenomenon or if it is restricted to specific conditions. Therefore, we transformed a *AtPII* genomic sequence that encodes a C-terminal GFP fusion and is

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under control of a ubiquitin promotor (*pUBQ10::AtPII*$_{genomic}$*-GFP*) into *Arabidopsis thaliana* Col-0 to observe the *At*PII localization under different conditions. Then we tested the impact of different temperature and light regimes on the *At*PII localization in this line. As it can be seen in Figure 2 differences in formation of PII foci can be observed depending on temperature or changing light quality. In this experiment most chloroplasts displayed GFP fluorescence also in the whole organelle. But nevertheless, PII foci of different sizes formed under all tested conditions to different extent without a clear recognizable trend (Figure 2).

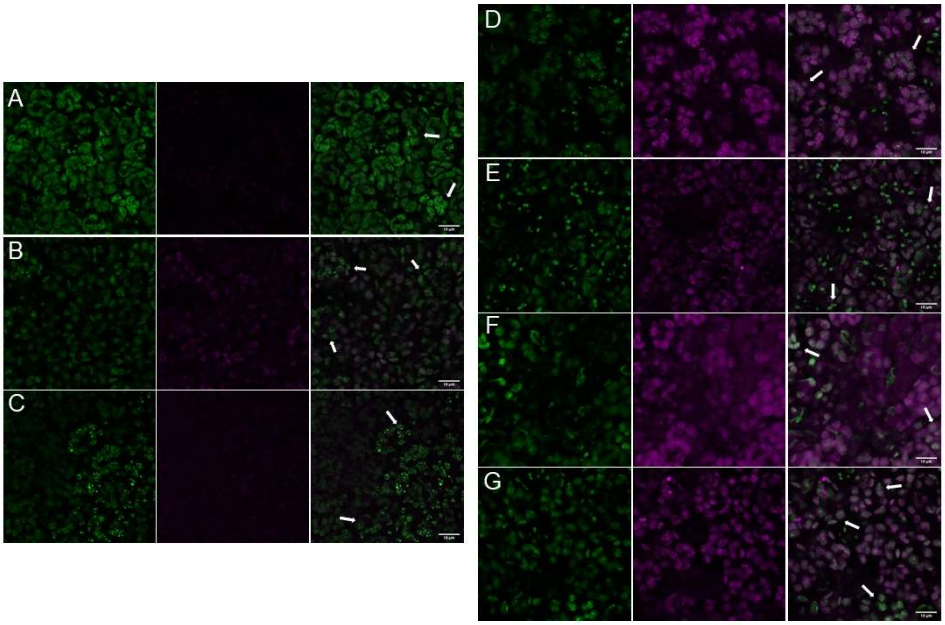

Figure 2. PII foci formation in *Arabidopsis thaliana* under different temperature and light regimes. Expression of genomic *At*PII-GFP under the control of the *pUBQ10* promotor (*pUBQ10::AtPII*$_{cDNA}$-GFP) was analysed in 6-day old seedlings. Seedlings were incubated for 24 h for temperature treatment in the dark at A) RT, B) 8°C, C) 37°C, and for light treatment in D) blue light, E) green light, F) red light, and G) far red light. Seedlings were fixed after incubation. In each row first the GFP fluorescence, second chlorophyll autofluorescence, and last the merge of both pictures is shown. White arrows: PII foci. Scale bar: 10 µm.

2.2. AtPII interaction with itself and other proteins takes place in PII foci

Next, we were interested if PII foci also appear when *At*PII interacts with itself or with other proteins. Therefore, we applied bimolecular fluorescence complementation (BiFC) to analyse physical interaction of the candidate proteins. It can be seen in Figure 3a that co-expression of *At*PII fused with nYFP and cYFP in tobacco leads to dot-like fluorescence as observed before. As *At*NAGK and *At*BCCP1 were characterized as PII interactors before, we cloned also both coding sequences to the same 2in1 BiFC vector together with *At*PII. Also, in these cases focal aggregation of fluorescence appeared after infiltration (Figure 3b, c).

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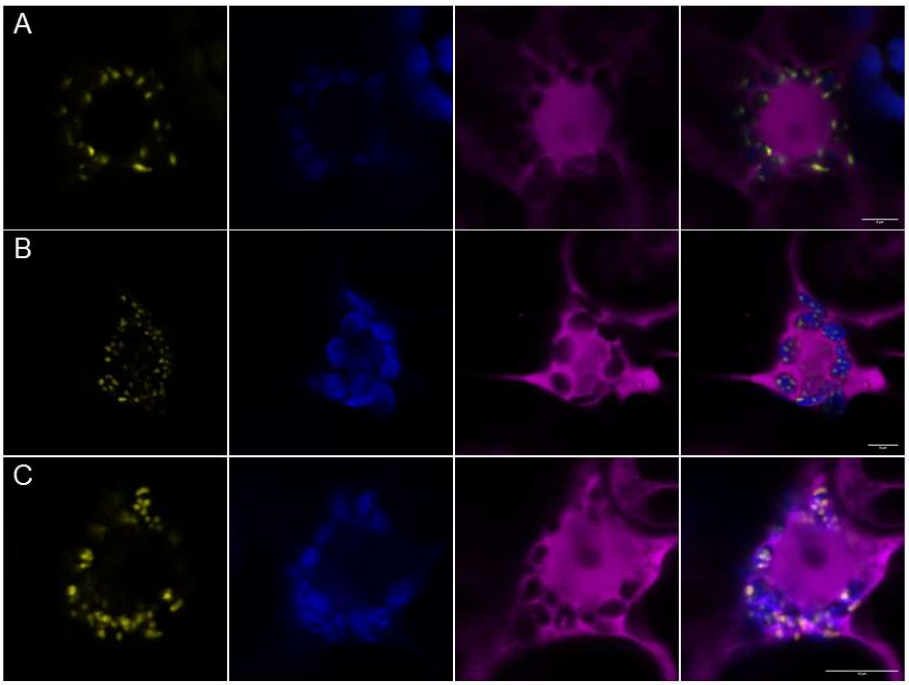

Figure 3. BiFC analysis of *At*PII with itself and known interactors in PII foci. *At*PII-nYFP was co-expressed with *At*PII-cYFP (A), *At*NAGK-cYFP (B), and *At*BCCP1-cYFP (C), respectively, under the control of *p35S* promotor using 2in1-BiFC vectors. Images were taken 3 days after transient transformation of *N. benthamiana* leaves. In each row first the YFP fluorescence (yellow), second chlorophyll autofluorescence (blue), third free RFP fluorescence as expression control (magenta) and last the merge of all pictures is shown. Scale bar: 10 µm.

These observations implied that the interaction of *At*PII with itself and other proteins takes place in PII foci. To confirm this finding, we co-expressed *At*PII and its putative interacting proteins as C-terminal fusions to GFP and mCherry, respectively, in suitable 2in1 vectors. Then, these vectors were infiltrated in tobacco and FRET/FLIM analyses were applied to co-localizing fluorescent signals. In Figure 4a-c it can be seen that co-localization of GFP and mCherry signals were observable in all experiments in dot-like structures. Fluorescence lifetime measurements (FLIM) revealed significantly reduced GFP fluorescence lifetime values of the co-localizing signals in comparison to *At*PII-GFP alone (Figure 4d). This observation confirms the assumption of physical interaction of *At*PII with itself and other proteins in PII foci, as such a reduction of fluorescence lifetime just occurs when two fluorescent proteins are in a critical distance of <10 nm [25].

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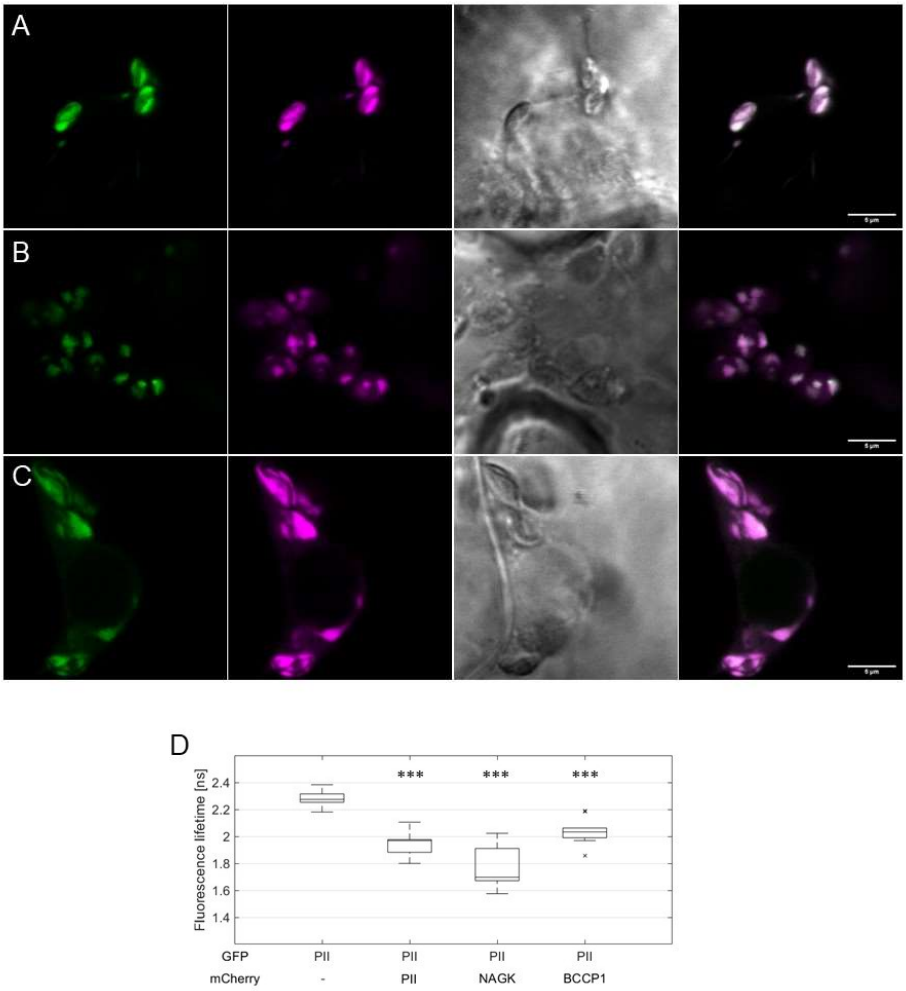

Figure 4. FRET/FLIM analysis of *At*PII interaction with candidate proteins in PII foci. *At*PII-GFP was co-expressed with *At*PII-mCherry (A), *At*NAGK-mCherry (B), and *At*BCCP1-mCherry (C), respectively, under the control of *p35S* promotor in *N. benthamiana* using 2in1 FRET vectors. Images were taken 2 days after transient transformation of *N. benthamiana*. In each row first the fluorescence of GFP, second of mCherry (magenta), third the brightfield image and last the merge of both fluorescence pictures is shown. Scale bar: 10 µm. D) FLIM analyses of fluorescent co-localizing signals in A) – C). Student's t-test used for calculation of significance. *** $p<0.001$.

Further analysis of *At*PII-GFP together with *At*NAGK-mCherry additionally revealed that the formation of PII foci evolves within seconds and that they just persist temporarily. In Figure 4 series of images of such an interaction over a time range of more than 2 min is shown. As it can be seen in this figure some of the PII foci evolve and vanish within a minute whereas others are visible over the whole range of time. Further analyses of PII foci showed that some of them are even stable more than half an hour (Figure S1).

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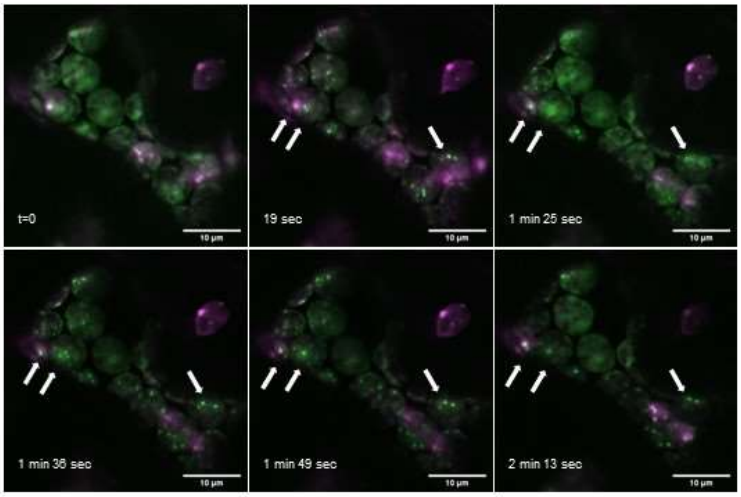

Figure 5. Appearance of PII foci over time. Overlay images of time series of *At*PII-GFP (green) and *At*NAGK-mCherry (magenta), both expressed under the control of *p35S*, 2 days after transient transformation of *N. benthamiana.* White arrows mark exemplarily PII foci observable over the whole time range. Scale bar: 10 µm

2.3. Characterization of PII foci points to plastidic protein degradation

Detecting *At*PII in focal aggregates in plastids opened the question if these suborganellar structures have been characterized before. As one candidate structure in plastids, it was tested if PII foci are part of nucleoids. Therefore, DNA in chloroplasts was visualized with YO-PRO1™ iodide as described previously [26]. In all tobacco cells expressing *At*PII-RFP this fluorescent signal could be clearly distinguished from the one of YO-PRO1™ iodide (Figure S2), which indicates that PII foci are not part of nucleoids in plastids.

Subplastidial compartments, similar to PII foci, were also reported to represent vesicle-like structures for protein degradation from chloroplasts [27, 28]. The small subunit of Rubisco (RBCS) is known to be part of such plastidial protein degradation vesicles, the Rubisco containing bodies (RCBs) [27, 29, 30].

To test if PII foci are part of the plastidic protein degradation apparatus, we co-expressed *At*PII-GFP with *At*RBCS3B-RFP in tobacco cells. Co-localization of the fluorescent signals in chloroplasts (Figure 6a) as well as decreased GFP lifetime in FLIM analyses (Figure 6d) confirmed our assumption. Furthermore, some of the co-localizing fluorescent foci could be found outside of chloroplasts (Figure 6a), supporting the assumption that *At*PII is part of plastidial protein degradation vesicles. To back up this hypothesis we tested also other proteins involved in autophagy-dependent protein degradation of RCBs [27, 31]. For this purpose, TagRFP or RFP tagged *At*NBR1, *At*g8e and *At*g8g from *A. thaliana* were co-expressed with *At*PII-GFP [32]. In particular cases co-localization of GFP and RFP signals could also be observed (Figure S3a-c).

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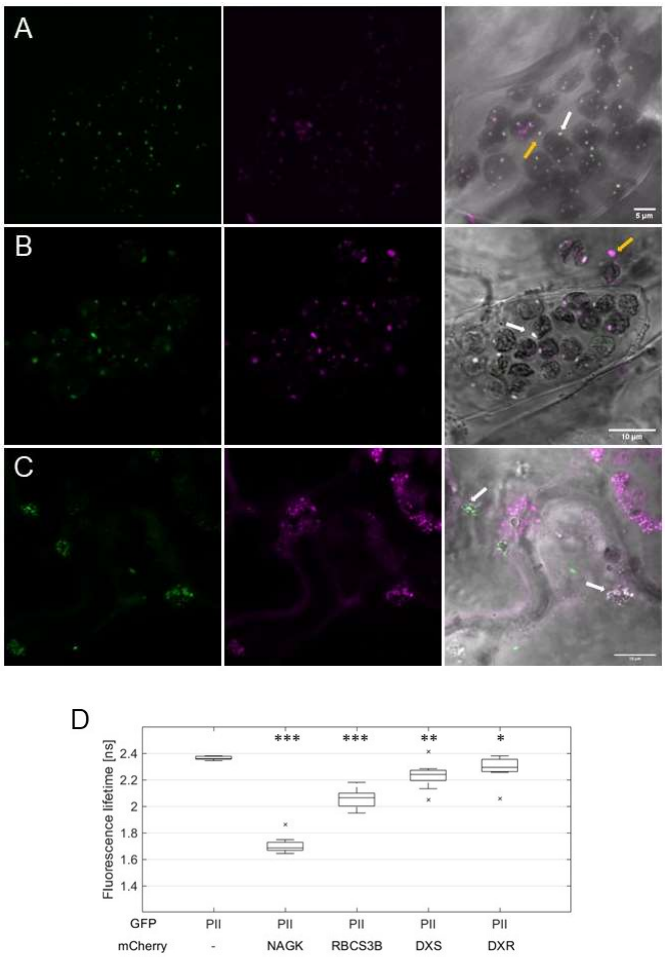

Figure 6. *At*PII is found in different plastidial aggregates. *At*PII-GFP was co-expressed with *At*RBCS3B-mCherry (A), *At*DXR-mCherry (B), and *At*DXS-mCherry (C), respectively, under the control of *p35S* in *N. benthamiana*. In each row first the GFP fluorescence is shown, second the mCherry fluorescence, and last the merge of both fluorescence images with the brightfield image as background. White arrows mark exemplarily AtPII aggregates in chloroplasts (dark and round structures in the brightfield image), orange arrows indicate extraplastidic vesicle-like structures. D) FLIM analyses of fluorescent co-localizing signals in A) – C) together with *At*PII-GFP/*At*NAGK-mCherry as positive control. Student's t-test used for calculation of significance. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Co-localization of *At*PII with proteins mediating different protein degradation pathways led to the assumption that *At*PII may be involved even in earlier steps of this process such as protein quality control (PQC). Previously, it could be shown that two enzymes of the methylerythritol-4-phosphate (MEP) pathway, deoxyxylulose-5-phosphate synthase (DXS) and reductoisomerase (DXR), undergo such PQC in *Arabidopsis* chloroplasts by aggregation, similar to PII foci [33, 34]. To test if *At*PII is also involved in PQC like *At*DXR and *At*DXS, they were also cloned into suitable 2in1 vectors together with *At*PII for fluorescence microscopy and subsequent FRET/FLIM analyses in tobacco after infiltration. The microscopic images revealed partial co-localization of *At*DXR and *At*DXS with PII foci (Figure 6b and c). Additionally, in FRET/FLIM analyses of co-localizing fluorescent signals, a significantly significant decrease of fluorescent lifetime was detected, indicating *in vivo* physical interaction of *At*PII with both *At*DXR and *At*DXS (Figure 6d).

2.4. C-terminus of AtPII is responsible for the formation of PII foci

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The observation that PII is found in plastidic protein degradation aggregates opened the question if this protein is just found there due to co-degradation in PQC, or if PII is indeed mediating the aggregation with other proteins. Furthermore, we were wondering about the differing size of the PII foci, which we observed in our microscopic studies. We assumed that a targeted change of PII protein structure could lead to differences in PII foci formation. To test this hypothesis, we decided to create a C-terminally truncated version of *At*PII without the so-called Q-loop. Almost all plant PII proteins possess a Q-loop motif at their C-terminus, which is the structure responsible for glutamine binding. The Q-loop affects the conformation of the T-loop in plant PII proteins, which is responsible for the interaction with target proteins [13].

Therefore, an additional version of *AtPII-GFP* without the Q-loop was cloned, in which the last 15 amino acids (*AtPII*$_{\Delta CT15}$*-GFP*) were deleted. For further analyses we co-expressed *At*PII-GFP or *At*PII$_{\Delta CT15}$-GFP with *At*PII-mCherry, *At*NAGK-mCherry and *At*RBCS3B-mCherry into tobacco leaves, each. To test the physical interaction of *At*PII-GFP and *At*PII$_{\Delta CT15}$-GFP with the mCherry tagged interactors, first the GFP tagged proteins were isolated from protein extracts with GFP traps. Afterwards, the co-eluted proteins were detected in a western blot with an αRFP antibody. As it can be seen in Fig. S4, infiltration of either *At*PII-GFP or *At*PII$_{\Delta CT15}$-GFP led to co-purification of all co-infiltrated interactors. These results indicated that *At*PII$_{\Delta CT15}$-GFP is still able to interact with itself and its binding partners.

In contrast, fluorescent microscopic analyses of these co-infiltrations revealed differences between the *At*PII variants in terms of PII foci formation: Co-infiltrated with *At*PII-GFP the number of PII foci is higher and their size smaller in comparison to *At*PII$_{\Delta CT15}$-GFP (Figure 7A-F). Additionally, with *At*PII$_{\Delta CT15}$-GFP the GFP signals were not confined to PII foci, but tend to spread within the whole chloroplast (Figure 7D-F).

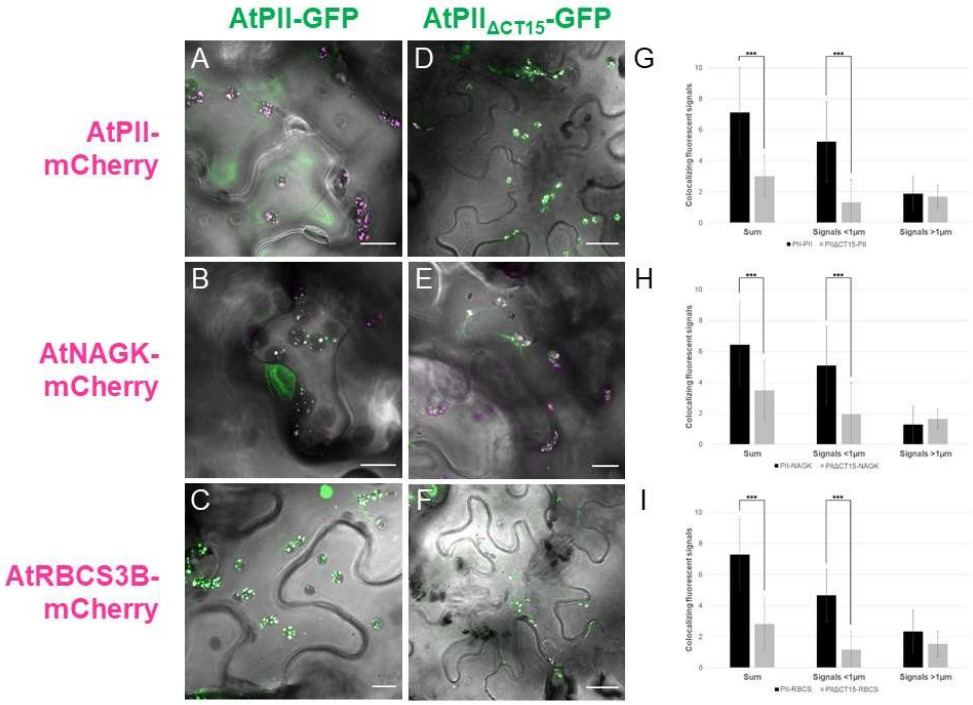

Figure 7. Truncation of the C-terminus of *At*PII leads to different aggregation behaviour. *At*PII-GFP or *At*PIIΔCT15-GFP were co-expressed either with *At*PII-mCherry (A, D respectively), *At*NAGK-mCherry (B, E respectively) or *At*RBCS3B- mCherry (C, F respectively), under the control of p35S in *N. benthamiana*. Each of these pictures shows the merge of GFP and mCherry fluorescence with the brightfield image as background. Size bars show 10µm except for D and F, where they indicate 22µm. In G-I the average total number (Sum) and the number of small and large co-localizing fluorescent signals per chloroplast for the co-expressions of *At*PII-

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GFP/*At*PII$_{\Delta CT15}$-GFP + *At*PII-mCherry (G), + *At*NAGK-mCherry (H) and *At*RBCS3B-mCherry (I) are given (n = 25). Student's t-test used for calculation of significance. *** p<0.001.

This observation was supported by quantitative analysis of these images. Therefore, we differentiated and counted the co-localizing fluorescence signals according to their size in signals smaller than 1µm and larger ones. In Fig. 7G-I the number of co-localizing fluorescence signals per plastid is given. It gets obvious that truncation of the C-terminus of *At*PII leads to a significant reduction of PII foci number in all tested cases. Specifically, the number of small signals decreases significantly in chloroplasts expressing *At*PII$_{\Delta CT15}$-GFP, whereas the number of large signals does not differ much (Figure 7G-I).

3. Discussion

In the present study it could be shown that *At*PII, either alone or co-expressed with its interaction partners, was almost exclusively found in aggregates in the chloroplasts. This aggregation, coined as PII foci in this study, took place irrespective of the promotor driving *At*PII expression or in which plant species (*Arabidopsis* or *Nicotiana*) it was expressed (Figures 1 and 2). This impression was even supported by the microscopic images of BiFC experiments of *At*PII interaction with itself, where the fluorescent signals were almost strictly confined to foci (Figure 3a).

As the physiological function of the PII protein in plants is still not clearly resolved, the appearance of such a characteristic structure like the PII foci raised several questions. PII proteins had been characterized in plants for the first time more than 20 years ago [7]. An obvious question in regard of PII foci is the one if this phenomenon had not been observed since that before. Revisiting former localization studies of plant PII proteins revealed that the aggregation of this protein in plastids had been indeed observed before, but did not attract further interest: In *Arabidopsis* cells PII was detected for the first time in chloroplasts by immunolocalization in a dotted pattern [35]. GFP fusions of two PII isoforms from maritime pine (*Pinus pinaster*) expressed in tobacco cells also revealed a similar localization pattern [36]. Focal aggregation of PII has even been observed in evolutionary distant organisms like the cyanobacterium *Synechococcus elongatus* [37]. These reports indicate that the formation of PII foci is an evolutionary conserved phenomenon and is not limited to *At*PII due to the slightly aberrant structure of the C-terminus without the glutamine binding site [13].

The presented results from our FRET/FLIM analyses, BiFC and co-immunoprecipitation experiments strongly argue in favour of direct interaction between *At*PII and the tested proteins within PII foci. But final evidence for physical interaction of *At*PII was just given for *At*NAGK [9, 38], whereas physical binding analyses with methods like surface plasmon resonance (SPR), isothermal calorimetry (ITC) or microscale thermophoresis (MST) for all other binding partners are still lacking. But the type of interaction may still be indirect as it has been shown for bacterial PII-PipX complexes to interact with the transcription factor PlmA [39], although a PipX-like protein has not been shown in plants to date. Nevertheless, the results of our studies revealed close proximity (approximately < 10 nm) between *At*PII and the tested proteins, which make a regulatory influence of *At*PII on neighbouring proteins very likely.

Our experiments of AtPII$_{\Delta CT15}$-GFP trap analysis indicated that the C-terminal Q-loop seems not to be essential for the binding of PII to its interactors although it has been suggested before that this region stabilizes the T-loop of AtPII and thereby the binding to AtNAGK [13]. This contradiction could be explained by the different sensitivity range of the analytics, where the biophysical methods such as SPR fail to detect weaker interactions [40]. The fact that formation of PII foci requires the Q-loop indicates that PII-target complex formation may not be the driving force in foci formation, but rather indicates a regulatory role of the C-terminus of AtPII for the formation of PII foci. This region was shown to be responsible for glutamine binding of plant PII protein with the evolutionary peculiar exception of its homologs in Brassicaceae [13]. In this regard it would be highly

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interesting to repeat these experiments with PII homologs from other plants, especially with C-terminally truncated versions, to investigate if there is a general correlation between glutamine, nitrogen supply and formation of PII foci. Also, the aggregation and disintegration dynamics of PII foci (Figure 5 and S1) in response to light, temperature and nutrient supply should be analysed in more detail, which may give further valuable information about the role of PII proteins in these processes.

The observation of PII foci leads to the greater question if this structural feature can be related to a physiological function. As it has been described in chapter 2.3 one putative function of PII in plants may be its contribution to plastidic protein degradation. This was deduced due to its subcellular co-localization and interaction to different components of the plastidic protein degradation pathways (Figure S3) and the observation of PII foci outside of chloroplasts (Figure 6a and b). Further analyses indicated physical interaction of *At*PII in PII foci to *At*DXR and *At*DXS, which are known to aggregate in course of inactivation and degradation [33, 34]. The accumulation and aggregation of both proteins is initiated and regulated by the so-called chloroplast unfolded protein response (cpUPR). In this process different Clp proteases and heat shock proteins (HSPs) are employed to regulate levels of proteins like *At*DXR and *At*DXS [34]. Furthermore, pharmacological and genetic approaches unraveled a crucial role of plastome gene expression (PGE) in cpUPR [34]. Our results of *At*PII interacting with *At*DXR and *At*DXS in PII foci imply an involvement of PII in cpUPR. This assumption is supported by the experiments with the C-terminally truncated *At*PII variant *At*PII$_{\Delta CT15}$-GFP: Infiltration of this construct together with *At*PII-, *At*NAGK-, or *At*RBCS3B-mCherry, led to diffusion of PII foci (Figure 7 and 8). This is reminiscent of images of *At*DXS-GFP expressing *Arabidopsis* plants either treated with an inhibitor of PGE or mutated in plastidic ribosome formation. In both cases also the dotted aggregation pattern of *At*DXS-GFP dispersed [34]. A putative function of PII in cpUPR would deliver a possible explanation why so far, the role of this protein in plants remained largely cryptic. Several different anabolic pathways like synthesis of tetrapyrrole, chlorophyll, carotenoids and isoprenoids have been identified to be prone to regulation of protein aggregation and degradation by cpUPR (for a summary see [41]). These pathways were not in the focus of the investigation of PII regulated processes, yet.

It is noteworthy in this regard, that in this study several novel interactors of a PII protein from plants have been reported. In addition to well characterized interactors like *At*NAGK, *At*BCCP1 and 2, and *At*BADC1-3 [12] the number of proteins as well as their functional range seems to be growing. This resembles the situation found for bacterial PII homologs and their interaction partners: For the PII homolog GlnZ from *Azospirillum brasilense* 37 interaction partners could be identified in ligand fishing assays [4]. In the cyanobacterium *Synechocystis* sp. all major transporter proteins involved in ammonium, nitrate and urea transport interact with the bacterial PII homolog GlnB [42]. Most interestingly, just NAGK could be identified as a common interactor in both of the studies, whereas all other proteins were functionally different [4]. Therefore, it seems reasonable that the list of this network in bacteria cannot be closed so soon. With the novel *Arabidopsis* PII interactors reported here it seems that PII interaction networks with many diverse protein partners are also an evolutionary conserved property in higher plants. However, these novel interactors may have moderate affinity and therefore escaped identification by co-purification experiments [12].

Nevertheless, even the actually characterized PII network in plants implies multiple regulatory functions of plant PII proteins. Interestingly, localization studies of *At*NAGK alone also revealed a dotted distribution in the *Arabidopsis* chloroplast like PII foci [43]. The same was observed for *At*BADC1, a protein which facilitates physical interaction of the PII interactor *At*BCCP1 within the htACCase [44]. It was further mentioned in this report that several other proteins from *Physcomitrella* and potato involved in lipid metabolism had also been observed in foci in chloroplasts [45, 46]. Furthermore, the colleagues noticed the similar subcellular distribution of PII proteins [44]. In this regard plastidic enzymes of lipid metabolism may be additional candidates of the PII foci network, which need to be confirmed in the future.

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Regardless of the physiological functions of PII foci the question awaits to be answered if PII is central for these suborganellar structures. Therefore, many of the presented subcellular localization studies have to be repeated in *Arabidopsis* in an *AtPII* null background like PIIS2, an *AtPII* knock-out line which had been isolated and described previously [19]. In the present report most of the results with *At*PII have been achieved in a heterologous system (*N. benthamiana*) with an endogenous PII encoding gene, whose impact on different regulatory phenomena must not be neglected as it can be done in a null background system. It would be highly interesting to express proteins like NAGK or BCCP from *Arabidopsis* alone and in conjunction with *At*PII in an *AtPII* knock-out line to see if the focal aggregation of PII interactors is directly depending on PII in plants.

Altogether, it can be stated that the presented results unravelled novel interactors of *At*PII proteins, which led to novel physiological processes regulated by PII proteins in plants. Although the regulatory mode is still unclear, the aggregation of *At*PII together with its interactors point to different possibilities of regulatory functions: A regulatory role of PII in plastidic protein degradation has to be taken into account as one possibility. But the presented data in regard of aggregation dynamics and proportion of extraplastidic PII foci make an exclusive role in this respect less probable. Another possibility to be tested in this regard should be the role of plant PII proteins in the formation of multi-enzyme assemblies or metabolons, which are known to be central to substrate channelling and metabolic regulation also in plants (for a summary see [47]). As PII proteins could bind to a variety of metabolic enzymes as mentioned above, an involvement of PII as a scaffolding protein is possible. Nevertheless, finding answers to these questions will be one of the central tasks for the elucidation of the physiological functions of PII proteins in plants.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

For transformation of *Arabidopsis thaliana* (Col-0) plants were grown on T- and R-soil mixed with sand (10:10:1) under long day conditions (16h light at 18°C, 8h dark at 15°C) at a humidity of 55-60% in the greenhouse. Stable transformed lines were generated using the floral dipping method according to [48].

Syringe mediated infiltration [49, 50] was used for transient transformation of leaves of three to four-week-old *N. benthamiana* with *A. tumefaciens* carrying plasmids of interest. Growth of *A. tumefaciens* and infiltration was performed according to protocol described in [51] derived from protocols of [49, 50, 52, 53] with the modification that cells were not washed with sterile H_2O before resuspension in AS medium.

For specific light and temperature treatment of *Arabidopsis* plants seeds were sown on ½ MS media (Murashige and Skoog basal salt, DUCHEFA Biochemie B. V. (Haarlem, Netherlands)). After stratification for one night at 4°C, plates were transferred for one day to constant light at 23°C, and placed in black boxes for additional three days in constant light at 22°C. One plate per condition was placed for 24h for temperature treatments in dark at 8°C, 23°C, and 37°C, and for light treatments in blue light (BL), green light (GL), red light (RL), and far red light (FRL). For further microscopic analysis, harvested seedlings were pre-fixed in 2 X SSC + 4% formaldehyde for 4 h, followed by vacuum infiltration for three times for 15 sec, and an additional incubation step for 30 min. Seedlings were transferred to 6-well plates and washed one time in 2 X SSC overnight, and twice for 1h. Seedlings were mounted on dH_2O on microscope slides and covered with cover glasses. Microscopic analysis was performed at Zeiss LSM880. Growth conditions and light treatment were modified according to protocol by [54].

4.2. Generation of Plant Expression Vectors

Coding DNA sequences (CDS) of *At*PII (AtGLB1-Start, AtGLB1-End), *At*PII$_{\Delta CT15}$ (AtGLB1-Start, AtPII-C2A), *At*NAGK (NK_AtNAGKstart, NK_AtNAGKend), *At*BCCP1 (NK_AtBCCP1start-2, NK-BCCP1end), and *At*RBCS3B (NK_RGCS1A-FP; NK_RGCS1A-

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RP) were amplified from cDNA of *Arabidopsis thaliana* Col-0 seedlings (for primer sequences see Table S1) for cloning into pENTR™/D-TOPO® (pENTR™ Directional TOPO® Cloning Kits from Invitrogen (Carlsbad, USA)) followed by LR (LR clonase, Invitrogen (Carlsbad, USA)) into either pUBQ10-Dest [55], pH7FWG2,0-Dest or pB7RWG2,0-Dest ([56]; for specifications of Vectors see Table S2).

Genomic constructs of the endogenous *AtPII* promoter (-269 bp) together with the genomic coding sequence of *At*PII or the genomic coding *At*PII sequence only were amplified using NK_proAtPIIstart/AtGLB1-End or AtGLB1-Start/AtGLB1-End, respectively, on genomic DNA extracted from *Arabidopsis thaliana* Col-0 followed by cloning into pENTR™/D-TOPO®. These entry constructs were cloned into pMDC107[57] or pUBQ10-Dest[55], respectively, by LR clonase reaction.

For the generation of 2in1 constructs for BiFC and FLIM analysis CDS of genes harbouring either P3P2 or P1P4 attachment sites were amplified, followed by BP reaction (BP clonase, Invitrogen (Carlsbad, USA)) into pDONR221-P3P2 and pDONR221-P1P4 (Invitrogen (Carlsbad, USA)), respectively, and LR reaction into pBiFCt-2in1-CC and pFRETgc-2in1-CC. Following primer combinations were used (for primer sequences see Table S1): AtPII P2P3 (NK_attP2P3-PIIstart; NK_attP2P3-PIIend), AtPII P1P4 (NK_attP1P4-PIIstart; NK_attP1P4-PIIend), AtNAGK P1P4 (NK_attP1P4-NAGKstart; NK_attP1P4-NAGKend), AtBCCP1 P1P4 (NK_attP1P4-BCCP1start; NK_attP1P4-BCCP1end), AtRBCS3B (NK_RGCS1A-P1P4-FP; NK_RGCS1A-P1P4-RP), AtDXS (NK_attP1-FP-DXS; NK_attP4-RP-DXS), AtDXR (NK_attP1-FP-DXR; NK_attP4-RP-DXR). pENTR-L1-GentR-L4 was used for the generation of the donor-only controls for BiFC and FLIM analysis by multisite LR in pBiFCt-2in1-CC and pFRETgc-2in1-CC together with pENTR-L3L2-PIIend.

4.3. Microscopic analyses

Imaging of fluorophores was performed on Leica TCS SP8 AOBS FLIM and Zeiss LSM880 Airyscan with a 63X/NA1.2 water objective. GFP and YO-PRO™-1 iodide were excited at 488 nm, and YFP at 514 nm using an Argon laser. RFP, mCherry and Chlorophyll were excited at 561 nm using a DPSS 561 nm laser.

YO-PRO™-1 iodide (Thermo Fisher Scientific (Waltham, USA)) staining was modified from [26]. *N. benthamiana* leaves were transiently transformed with *A. tumefaciens* harbouring AtPII-RFP under the control of *p35S* together with *A. tumefaciens* harbouring P19 or *A. tumefaciens* harbouring P19 alone. Leaf disks were cut out three days after infiltration, incubated in 2 X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) +/- RNase A (10 µg/mL), or 2 X SSC + 1 X DNase I buffer + DNase I (100 U/mL), for 4 h at 37°C, transferred to 2 X SSC + 4% formaldehyde. Leaf disks were vacuum infiltrated for 15 sec at 300 mbar for three times followed by incubation for 15 min. Washing was performed three times in 2 X SSC. Leaf disks were stained with DAPI (1 µg/mL) + YO-PRO™-1 iodide (5 µg/mL) in 2 X SSC overnight and washed 1 X with 1 X SSC. Leaf disks were mounted on microscopic slides on SSC/glycerol (50 % 2 X SSC + 50 % glycerol) and covered with cover slips. Confocal imaging was performed with Zeiss LSM880 Airyscan.

BiFC analyses were performed according to a modified protocol of [58] three days after transient transformation of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens* harbouring 2in1 *pBiFC* vectors of interest. Internal RFP fluorescence was used as transformation control only.

FRET-FLIM measurements were performed with Leica TCS SP8 AOBS FLIM equipped with SymPhoTime software (PicoQuant GmbH (Berlin, Germany)) and PicoHarp 300 (PicoQuant GmbH (Berlin, Germany)) for FLIM measurements and TimeHarp 260 Nano (PicoQuant GmbH (Berlin, Germany)) for rapidFLIM measurements. Measurements were performed of transiently transformed leaf disks of *N. benthamiana* leaves with *A. tumefaciens* GV3101 carrying 2in1 *pFRET* vectors of interest. FLIM measurements were performed according to [59] and [51]. Two biological replicates (FLIM and rapidFLIM) were measured in five to six regions (FLIM) and four to five regions (rapidFLIM) containing plastids in the epidermis. For FLIM measurements acquisition

was performed until 700 photons in the brightest point were counted. For rapidFLIM measurements acquisition was performed until 1500 photons in the brightest point were counted.

All Images acquired were processed using Fiji (Fiji is just ImageJ; [60], based on ImageJ) and Microsoft Office 2019 PowerPoint (Microsoft Corporation (Redmond, Washington, USA).

4.4. Statistical analyses

Statistical analyses of FLIM measurements using two-sided Student's t-test as well as generation of box plots were done in Matlab (The MathWorks Inc. (Natick, USA)). Statistical analyses of PII foci numbers were performed using two-sided Student's t-test in Microsoft Excel.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: PII foci can be stable for more than 30 min, Figure S2: PII foci are not nucleoids, Figure S3: PII co-localizes partially with autophagy-related proteins, Figure S4: AtPII$_{\Delta CT15}$-GFP can also bind to its interactors Table S1: Primers used for the construction of the different plant expression vectors. Table S2: Vectors used for the construction of the different plant expression vectors.

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