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SUMOylation of PHYTOCHROME INTERACTING FACTOR 3 promotes photomorphogenesis in *Arabidopsis thaliana*

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SUMMARY

- In *Arabidopsis thaliana* phytochrome B (phyB) is the dominant receptor of photomorphogenic development under red light. phyB interacts with a set of downstream regulatory proteins, including PHYTOCHROME INTERACTING FACTOR 3 (PIF3). The interaction of PIF3 and the photo-activated phyB leads to the rapid phosphorylation and degradation of PIF3 and also to the degradation of phyB, which events are required for proper photomorphogenesis.
- Here we report that PIF3 is SUMOylated on the Lys13 (K13) residue and we could detect this posttranslational modification in a heterologous experimental system and also *in planta*.
- We also found, that the SUMO acceptor site mutant PIF3(K13R) binds stronger to the target promoters than its SUMOylated, wild-type counterpart. Seedlings expressing PIF3(K13R) show elongated hypocotyl response, elevated photoprotection and higher transcriptional induction of red light responsive genes compared with plantlets expressing wild-type PIF3.
- These observations are supported by the lower level of phyB in those plants which possess only PIF3(K13R) indicating that SUMOylation of PIF3 alters photomorphogenesis also via the regulation of phyB amount. Conclusively, whereas SUMOylation is generally

connected to different stress responses it also fine tunes light signalling by reducing the biological activity of PIF3 thus promoting photomorphogenesis.

KEY WORDS

Arabidopsis thaliana, light signalling, PIF3, phytochrome, photomorphogenesis, SUMO, SUMOylation

INTRODUCTION

Light sensing plant photoreceptor molecules are responsible for light perception and initiating signalling responses necessary for survival and achieving optimal fitness in the ever-changing light environment. There are five phytochromes (phyA-E) in Arabidopsis (*Arabidopsis thaliana*) which are responsible for red (R) and far-red (FR) light sensing (Nagy and Schafer, 2002; Legris et al., 2019). Among them phyB is the dominant and responsible for photomorphogenic development in R light. Phytochromes are synthesized in the inactive conformer (Pr), which is converted to the biologically active Pfr form upon R light illumination. Photoactivated phyB governs diverse signalling pathways and interacts with a set of bHLH transcription factors, PHYTOCHROME INTERACTING FACTORs (PIF) (Leivar and Quail, 2011). One of them, PIF3, is involved in repressing photomorphogenic development in the dark but promotes photomorphogenesis after the onset of light (Al-Sady et al., 2008; Leivar et al., 2008; Shin et al., 2009; Stephenson et al., 2009; Zhang et al., 2013). Light-activated phyB Pfr is accumulated in the nucleus, interacts with PIF3 and this leads to fast decrease of PIF3 protein levels (Ni et al., 1999; Bauer et al., 2004; Park et al., 2004). PIF3 is phosphorylated by several different kinases on multiple residues (Ni et al., 2013; Shin et al., 2016; Ling et al., 2017; Ni et al., 2017) and this posttranslational modification (PTM) is necessary for ubiquitination and subsequent degradation of the protein (Park et al., 2004; Al-Sady et al., 2006). It turned out that along fast PIF3 degradation, there are other mechanisms to decline the active PIF3 amount after the onset of light: *PIF3* transcript level is also decreased in light (Shi et al., 2016), phyB sequesters PIF3 and release it from a DNA target (Park et al., 2012; Park et al., 2018), and additionally a recent study demonstrated that phyB inhibits PIF3 translation by intron retention (Dong et al., 2020).

Interestingly, despite the fact that fast decrease of available PIF3 amount is one of the first molecular events of photomorphogenesis, PIF3 has role in hypocotyl and cotyledon growth under

light/dark photocycles (Soy et al., 2012); fine-tuning circadian responses (Soy et al., 2016); developing freezing tolerance (Jiang et al., 2017); regulating stomatal opening (Wang et al., 2010) and protecting young seedlings from reactive oxygen species (Chen et al., 2013). Additional reports indicate that PIF3 mediates hormonal responses and together with other PIFs, it is involved in the integration of light and hormonal signalling (Lau and Deng, 2010; Yang et al., 2012; Zhong et al., 2012; Zhang et al., 2014; Zhong et al., 2014; Li et al., 2016). It was also shown that PIF3 does not act alone but redundantly controls gene expression with other PIFs forming a signalling hub with diverse functions (Zhang et al., 2013; Leivar and Monte, 2014; Pfeiffer et al., 2014).

Arabidopsis expresses 4 isoforms of the Small Ubiquitin-like MOdifier (SUMO) proteins, SUMO1-3 and SUMO5 (Kurepa et al., 2003; van den Burg et al., 2010; Hammoudi et al., 2016). The attachment of SUMO is a posttranslational modification (PTM) among eukaryotes involving activation, conjugation and ligation of SUMO to lysine amino acids located in a conserved sequence motif of the target protein (Novatchkova et al., 2004; Park et al., 2011; Vierstra, 2012; Castano-Miquel et al., 2013; Tomanov et al., 2014; Augustine and Vierstra, 2018). SUMO and ubiquitin proteins show high structural similarities, but whereas ubiquitination typically directs the target protein to proteasomal degradation (Vierstra, 2009), SUMOylation has more diverse outcomes, e.g. changes in stability, enzyme activity, nuclear localization, protein interaction, etc. of the target protein (reviewed by (Augustine and Vierstra, 2018)). Tightly controlled enzymatic de-SUMOylation of the targets opens additional regulatory pathways (Yates et al., 2016). It was observed that the overall SUMOylation of the plant proteome is increased during various stress responses (e.g. heat, drought, salt, pests, etc.) and also under developmental changes (flowering, growth, etc.) (Kurepa et al., 2003; Murtas et al., 2003; Miura et al., 2005; Lee et al., 2007; Lee et al., 2007; Conti et al., 2008; Miller et al., 2010; Miller et al., 2013; Bailey et al., 2016; Cai et al., 2017; Castaño-Miquel et al., 2017; Rytz et al., 2018) (recently reviewed by (Castro et al., 2012; Elrouby, 2015; Elrouby, 2017; Augustine and Vierstra, 2018; Verma et al., 2018)).

Light signalling pathways have drastic impact on plant development and their protein components are also targets of SUMOylation. For example, SUMOylation of the available phyB pool is increased by red light and reaches high levels in the middle of the day in plants grown under diurnal conditions. It was also shown that phyB SUMOylation inhibits phyB-PIF5 binding and attenuates light signalling (Sadanandom et al., 2015). Not only the phyB photoreceptor but another important component of light signalling, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is also SUMOylated. This ubiquitin ligase has key function in maintaining etiolated

growth and preventing photomorphogenesis in darkness. SUMOylation increases the activity of COP1 and the fact that COP1 regulates the abundance of the SIZ1 SUMO ligase, links light signalling tightly to different stress responses mediated by SUMOylation (Kim et al., 2016; Lin et al., 2016; Hammoudi et al., 2018; Mazur et al., 2019).

A recent study showed that FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) a nuclear transporter of the photoactivated phytochrome A is SUMOylated and this modification accelerates FHY1 degradation (Qu et al., 2020). These results indicate that light signalling is modified by SUMOylation not only by targeting the photoreceptor but also downstream components of the signal transduction pathways.

Our data indicate that another key light signalling component and phyB direct interactor, PIF3 is also SUMOylated. We noticed that SUMOylation of PIF3 leads to decreased PIF3 activity, thus propagates cotyledon expansion and inhibition of hypocotyl elongation in R light and attenuates PIF3-mediated gene induction and photoprotection. These phenotypic responses can be the result of weaker DNA binding affinity of SUMOylated PIF3 and by the higher stability of phyB in those plants which contain SUMOylated PIF3. Our work suggests that in contrast to the previously identified PTMs, SUMOylation of PIF3 modulates the activity, rather than the available amount of this transcription factor.

MATERIALS AND METHODS

Plant materials

Throughout the study we used *pif3-3* (Monte et al., 2004) mutant of *Arabidopsis thaliana* (Columbia ecotype). Plant transformation was performed using the floral dip method and transgenic lines containing a single transgene locus were selected. *pifq/35S:PIF3-YFP* has already been published (Pfeiffer et al., 2012). *phyB-9* mutant (Reed et al., 1993) was used as a control in Fig 2A.

Molecular cloning

PIF3(K13R) was generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent) according to the instruction of the manufacturer. The coding sequences of the *PIF3* and *PIF3(K13R)* were fused to the coding region of the *YELLOW FLUORESCENT PROTEIN* (*YFP*) as *Bam*HI-*Sma*I fragments in the pPCV binary vector (Bauer et al., 2004). *PIF3* and *PIF3(K13R)*

coding regions were cloned as *Bam*HI-*Not*I fragments into the pET28 vector (Novagen) for bacterial protein expression. All clones were checked by sequencing. Supporting Table S1. shows the sequence of the oligonucleotides used for cloning.

Seedling phenotyping

Measurement of hypocotyl elongation and data evaluation was performed as described earlier (Adam et al., 2013; Dobos et al., 2019). In the survival test, seedlings were grown in the dark on Murashige-Skoog medium plates for 4 days and then were placed under white light irradiation (100 μmol m^{-2} s^{-1}) for 2 days. The ratio of total/survived seedlings was calculated. All experiments were repeated at least 3 times.

SUMO binding site predictions

The *in silico* SUMO binding site predictions for different PIF3 homologues were performed using the current version (as of 03. 05. 2020.) of the GPS-SUMO online tool at medium sensitivity (http://sumosp.biocuckoo.org, (Zhao et al., 2014). The alignment was done using CLUSTAL O (version: 1.2.4) at www.ebi.ac.uk. The GeneBank accessions of the used sequences are as follows: *Arabidopsis thaliana* (NP_001318964); *Eutrema salsugineum* (XP_006417565); *Citrus clementina* (XP_006423962); *Capsella rubella* (XP_006303172); *Eucalyptus grandis* (XP_010070103); *Aquilegia coerulea* (PIA60627); *Gossypium barbadense* (PPS10307); *Cucumis sativus* (XP_011648884); *Vitis vinifera* (RVW77362); *Cephalotus follicularis* (GAV72297); *Populus trichocarpa* (XP_006382253); *Physcomitrella patens* (XP_024361305); *Arachis duranensis* (XP_015939567).

Total plant protein extraction and immunoblotting

Details of total plant protein extract preparation and western blot analysis were described earlier (Vanhaelewyn et al., 2019). We used the following primary antibodies: anti-PIF3 (Bauer et al., 2004), anti-SUMO1 (Agrisera), anti-T7 (Novagen), anti-ACTIN (SIGMA-Aldrich), anti-PHYB (generous gift of Prof. Peter H. Quail, UC, Berkeley) and the following secondary antibodies: Polyclonal Swine Anti-Rabbit Immunoglobiulins/HRP (Dako), and Goat Anti-Mouse igG Peroxidase Conjugated antibody (Invitrogen). The signals were visualized using Immobilon Western HRP Substrate (Millipore) according to the recommendation of the manufacturer using a cooled digital camera (Hamamatsu Orca-II).

Immunoprecipitation of PIF3-YFP

This procedure has been described in detail (Orosa and Viczian, 2019). Shortly, 1 g of frozen plant material was ground in liquid nitrogen and mixed with 1.8 mL Extraction buffer (30 mM Tris-HCl pH=8.8, 1% SDS, 1% (w/v) Triton X100, 50 mM sodium-bisulfite, 20 mM N-Ethylmaleimide (Sigma), 1 mM phenylmethylsulfonyl fluoride (Roche), 1 piece/10 mL buffer cOmplete ULTRA Mini Protease Inhibitor tablet (Roche)). Thawed samples were collected into 2 mL reaction tubes and cleared with centrifugation (20000× *g*, 4 °C, 15 min). The supernatant was added to 30 µL anti-GFP agarose beads (Chromotec) equilibrated in extraction buffer. Samples were rotated in a roller drum for least 30 min (20 rpm, 4 °C), the supernatant was discarded and the beads were washed four-times with Extraction buffer. After washing, 30 µL 2xR Loading buffer (125 mM Tris-HCl pH=6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol-blue) was added to each sample. After 5 minutes of incubation at 95 °C, samples were centrifuged (20000× *g* 1 min) to settle down the agarose beads before loading the supernatant onto denaturing acrylamide gel.

Transcript level determination

Seedlings were surface sterilized, placed on Murashige-Skoog agar plates and were kept at 4 °C for 3 days. After 6 hours of white light irradiation (100 µmol m^{-2} s^{-1}, LUMILUX XT T8 L 36 W/865 fluorescent tubes, Osram) they were kept in darkness for 5 days at 22 °C. Seedlings were irradiated with 10 µmol m^{-2} s^{-1} red light (SNAP-LITE LED light source, Quantum Devices Inc.) for 60 min and snap frozen in liquid nitrogen. Total plant RNA was isolated using the Nucleospin Plant II Maxi kit (Macherey-Nagel), the reverse transcription reaction was performed using the RevertAid First Strand cDNA synthesis Kit (Thermoscientific), whereas the qRT-PCR reaction was made using the qPCRBIO Sygreen Mix (PCR Biosystems) according to the instruction of the manufacturers. Supporting Table 1. shows the sequence of the used oligonucleotides for the qRT-PCR assays. The mRNA levels are presented as relative to the constitutively expressed *TUBULIN2/3* mRNA transcript (Endo et al., 2007).

***In vitro* SUMOylation assay**

PIF3 pET28 and PIF3(K13R) pET28 constructions were transformed into *E. coli* BL21 strain containing plasmids for expressing the SUMO-activating (E1) and conjugating (E2) enzymes and one of the four SUMO isoforms (Okada et al., 2009)
10 mL LB cultures were incubated at 37 °C until reaching OD_{660}=0.6, then β-D-1-thiogalactopyranoside (IPTG, Biosynth AG) was added to reach the final concentration of 0.5 mM to each culture. The bacterial cultures were further incubated overnight in 16 °C. Cells were collected by centrifugation (3000× g, 4 °C, 20 min) and the pellet was re-suspended in 1 mL sterile water. 10 µL cell suspension was mixed in 20 µL 2xR Loading buffer and denaturated (95 °C, 10 min). We collected the debris by centrifugation (20000× g, 1 min) and then we immediately loaded 28 µL of the supernatant on denaturing polyacrylamide gel. PIF3 and PIF3-SUMO were detected by anti-T7 antibody.

Expression and purification of *in vitro* SUMOylated PIF3 for EMSA, Co-IP and MS/MS assays

PIF3-pET28 and PIF3(K13R)-pET28 constructions were transformed into *E. coli* BL21 strain containing plasmids for expressing the SUMO-activating (E1) and conjugating (E2) enzymes and one of the four SUMO isoforms (Okada et al., 2009). 1 L bacterial culture (in Luria Bertani broth) was incubated in 37 °C until OD_{660} reached value 0.6 then IPTG was added to reach the final concentration of 0.5 mM to the cultures and were incubated for 4.5 hours in 37 °C. Cells were collected and re-suspended in 12 mL Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole (PanReac AppliChem), pH: 8.0) and then 10 mg lysosyme (Fluka), one cOmplete ULTRA Mini Protease Inhibitor tablet, 20 mM N-Ethylmaleimide (NEM, Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Roche) were added to each sample. Samples were incubated on ice for 30 min followed by 12 times 10 s sonication. Supernatant was cleared by centrifugation (20000× g, 4 °C, 25 min) and loaded on 3 mL Ni-NTA Agarose beads (Qiagen), equilibrated in Lysis buffer. Samples were rotated on a roller drum for 1 hour in 4 °C (20 rpm). Beads were collected (1000× g, 4 °C, 2 min) and washed 3 times using ice cold Washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole). The proteins were eluted from the beads using 2 mL of Elution buffer (5 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole) 3 times and the samples were concentrated using Amicon Ultracel – 30K filter tubes (Merck) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)

To produce double-stranded probes, equal amounts of complementary oligonucleotides were mixed at a final concentration of 40 µM in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, heated to 95 °C for 5 min and let to cool down in a block heater to room temperature (RT) overnight. The 5' end of the forward oligonucleotide was labelled with biotin (Thermo Scientific). PIF3 and PIF3(K13R) proteins with an N-terminal 6xHis tag were expressed in *E. coli* BL21 cells and purified using Ni-NTA agarose matrix as the manufacturer recommended (QIAexpressionist, Qiagen,). In the binding reactions 10 mM Tris-HCl (pH=7.5), 85 mM KCl, 5% (v/v) glycerol, 0.1 µg/µL poly(dI-dC), 40 fmol probe and variable amount of *E. coli* expressed and purified PIF3 proteins were mixed in 20 µL volume. Reactions were incubated at RT for 20 min and loaded on native 4% polyacrylamide gels. Gels were run in 0.5× TBE and electro-blotted to nylon membrane (Hybond-N+, Amersham) in 0.5× TBE. Detection of biotin-specific signal was done using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer's instruction. Signals were detected as described for the Western-blots.

Accession numbers of Arabidopsis genes

PIF3: AT1G09530; *ELIP2*: AT4G14690; *CHS*: AT5G13930; *PHYB*: AT2G18790; *PIL1*: AT2G46970; *TUB2/3*: AT5G62700, AT5G62690

Details of mass spectrometry and yeast two-hybrid assays are available as Supporting Information Methods S1.

RESULTS

Identification of the SUMOylated residue in PIF3

We performed an *in silico* search to identify potential SUMO target residues in the Arabidopsis PIF3 protein. The GSP-SUMO software (Zhao et al., 2014) identified Lysine 13 (K13) as a potential SUMO attachment target with high probability as part of a conventional consensus site ψ–K–X–E/D (ψ = hydrophobic residue, X = any amino acid) (Hendriks and Vertegaal, 2016; Augustine and Vierstra, 2018). Furthermore, we also noticed that a lysine residue located in similar position is a potential SUMOylation target at the N-terminus of PIF3 among different plant species (Fig 1A). To demonstrate *in vitro* the PIF3 SUMOylation we expressed this protein in a bacterial system which reconstitutes Arabidopsis SUMOylation in *E. coli* by

expressing Arabidopsis SUMO proteins along with the necessary SUMO-conjugating enzymes (Okada et al., 2009). We found that (i) we can detect SUMO-PIF3 conjugates, (ii) all plant-expressed active SUMOs (SUMO1, SUMO2, SUMO3, SUMO5) were conjugated to PIF3, (iii) only the conjugatable SUMOs were attached to PIF3 and (iv) we could not detect SUMO conjugated to the PIF3(K13R) mutant in which the Lysine 13 was replaced by Arginine (Supporting Fig S1). Furthermore, we could also confirm the SUMOylation of Lysine 13 by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Supporting Fig S2).
To test PIF3 SUMOylation *in planta*, we fused PIF3 and PIF3(K13R) to the YELLOW FLUORESCENT PROTEIN (YFP) and expressed PIF3-YFP and PIF3(K13R)-YFP at high levels under the control of the constitutive viral *p35S* promoter in the *pif3* mutant background. To enrich the PIF3 content of our sample, and to get rid of other SUMOylated proteins, we immunoprecipitated PIF3-YFP and PIF3(K13R)-YFP using GFP-Trap agarose beads. Next we tested these samples with western blot analysis applying the anti-SUMO1 antibody. We found, that similarly to the *in vitro* bacterial system, PIF3 is also SUMOylated *in planta*, whereas we could not detect SUMO signal on the PIF3(K13R) mutant protein (Fig 1B). We also noted that PIF3-SUMO conjugates occur also in etiolated seedlings and in plantlets grown under light/dark cycles, and accumulates to higher levels in the dark phase (Fig 1B, Supporting Fig S3A).

SUMOylation reduces the biological activity of PIF3 in light

To examine the biological role of PIF3 SUMOylation, we expressed *p35S:PIF3-YFP* and *p35S:PIF3(K13R)-YFP* transgenes in *pif3* mutant background and chose those lines which express the chimeric proteins at the same level (Supporting Fig S3B-D). Afterwards, we measured the light-induced inhibition of hypocotyl elongation of these lines grown under constant R light, which assay is widely used to monitor the performance of phyB-mediated light signalling. We noticed that those plants which express the wild type (WT) PIF3 has longer hypocotyl in red light (hyposensitive photomorphogenic response), compared with the background *pif3* mutant plants (Fig 2A). This result recapitulates earlier observations (Kim et al., 2003; Bauer et al., 2004; Monte et al., 2004). More interestingly, those seedlings which express the SUMO acceptor site mutant PIF3(K13R) show even more hyposensitive hypocotyl elongation phenotype, despite PIF3-YFP and PIF3(K13R)-YFP proteins are expressed at the same levels. The inhibition of hypocotyl elongation response is even weaker in two other plant lines expressing PIF3-YFP and PIF3(K13R)-YFP proteins at higher but equal levels (Supporting Fig S3B) and they respond to

light only under higher fluences. We suspect, that the response is saturated and the high level of PIF3 expression masks the subtle differences between the lines (Fig 2A). PIF3 also plays a key role promoting hypocotyl growth and inhibiting cotyledon expansion in seedlings grown under light/dark photoperiods (Soy et al., 2012; Soy et al., 2016). Under these growth conditions, PIF3-YFP and PIF3(K13R)-YFP proteins are expressed in the transgenic plants at the same levels (Supporting Fig S3E-F). The longer hypocotyls and the smaller cotyledons of the plants expressing PIF3(K13R) indicate that PIF3 SUMOylation promotes seedling photomorphogenic growth under light/dark photoperiods similarly to under constant irradiation (Fig 2B-C).

It is well established, that PIF3 is required for proper chlorophyll accumulation and seedling greening. Lack of PIF3 results in over-accumulation of protochlorophyllide, a precursor of chlorophyll, causing photobleaching and cell death when etiolated seedlings are transferred to light (Shin et al., 2009; Stephenson et al., 2009; Chen et al., 2013). To test this response, we transferred etiolated seedlings to white light and 2 days later calculated the ratio of viable and dead seedlings. This survival rate was 78.2% in case of the WT plants but only 13.8% of the *pif3* mutant seedlings survived the light treatment. Expressing WT PIF3-YFP in the *pif3* background increases the number of survival plants dramatically and complements the mutant phenotype, whereas PIF3(K13R)-YFP having mutated SUMO acceptor site induces an even higher survival rate (Fig 2D). Those lines which express PIF3-YFP or PIF3(K13R)-YFP molecule at higher levels induce equally high response. Most probably, the response is saturated and subtle differences between the lines are masked in these high level expressors (Fig 2D).

Conclusively, these results indicate that the SUMO acceptor site mutant PIF3(K13R) molecules trigger stronger PIF3-mediated responses than the WT counterparts, in other words, SUMOylation reduces the biological activity of PIF3 in young seedlings in light.

Binding to target promoters affected by the SUMOylation of PIF3

Light-induced degradation in etiolated seedlings is a characteristic property of PIF3 (Bauer et al., 2004; Park et al., 2004). We found that both the PIF3-YFP and of PIF3(K13R)-YFP amounts decreased quickly after the onset of R light irradiation and no difference in the degradation of the two proteins can be detected, noting the obvious limitations of the applied western blot hybridisation analysis which may hide subtle temporal differences between the sample collection time points (Supporting Fig S4). Not only the light-induced degradation but also the intracellular localization and nuclear complex formation of PIF3(K13R)-YFP resembles of its

WT counterpart (Supporting Fig S5). Collectively, these results indicate that SUMOylation does not alter the light-induced degradation and intracellular localization of PIF3.

PIF3 transcription factor takes part in mediating light-induced gene expression. This response is among the first molecular mechanisms which interrupts etiolated growth and contributes to proper photomorphogenic development after the onset of light (Al-Sady et al., 2008). We examined the induction of two genes, *EARLY LIGHT-INDUCED PROTEIN 2* (*ELIP2*) and *CHALCONE SYNTHASE* (*CHS*) and found that a short R light treatment increases their expression level. This increase is higher in those plants which express PIF3(K13R)-YFP than of those of PIF3-YFP expressors at a very early stage of the photomorphogenic development after 1 h of R irradiation (Fig. 3).

Although the composition of protein complexes containing PIF3 and regulating transcription is obscure but the direct binding of PIF3 to certain target promoters and the strength of this binding can be examined with electromobility shift assay (EMSA). We expressed and isolated PIF3 and PIF3(K13R) from an *E. coli* strain which is not able to SUMOylate proteins and we did not find differences between the DNA binding of the WT and the K13R mutant (Supporting Fig S6A). This indicates that a single amino acid exchange does not alter PIF3 binding to DNA. Next, we expressed and purified PIF3 and PIF3(K13R) from *E. coli* cells which contained all necessary elements of the Arabidopsis SUMOylation pathway and used these to perform an EMSA with a G-box motif containing (CACGTG) promoter section of the *PIF3-like 1* (*PIL1*) which is shown to be a binding target of PIF3 (Zhang et al., 2013). Applying the same amount of WT and mutant protein we found that PIF3(K13R), mutated in the SUMO acceptor site, binds with higher affinity to the target DNA sequence (Fig 4, Supporting Fig S7). We received the same results when used the G-box containing promoter elements of *ELIP2* and *CHS* (Supporting Fig S8). Collectively, these results indicate that SUMOylation can modulate the binding affinity of PIF3 to different target promoters.

SUMOylation state of PIF3 affects the stability of PHYB

A few years ago it was revealed that not only the degradation of PIF3 is induced by light but PIF3 and PHYB interact in a protein complex and co-degrade (Ni et al., 2014). This process decreases the amount of available active photoreceptors thus fine-tunes photomorphogenesis. We wanted to examine how SUMOylation of PIF3 influences PHYB level in our transgenic lines expressing PIF3-YFP or PIF3(K13R)-YFP at equal levels. During continuous R light irradiation,

using western blotting we could detect less phyB in the line which express PIF3(K13R)-YFP (Fig 5A, Supporting Figure S9A) indicating that PIF3 SUMOylation stabilizes PHYB.

This observation can be explained by the different stability of the complex formed by the SUMOylated or by the SUMO acceptor site mutant PIF3 with phyB. We found that K13R mutation per se does not alter PIF3 binding to phytochromes (Supporting Fig S6B) and to test the effect of PIF3 SUMOylation on phyB binding, we expressed PIF3 in an *E. coli* strain which is able to SUMOylate proteins. Supporting Figure S7A-B shows, that not the whole available amount of PIF3 is SUMOylated in the bacterial cells, but a considerable amount of non-SUMOylated protein is also present. We used this mixture of PIF3 and SUMO-PIF3 to incubate together with native protein extract made from PHYB-YFP expressor plants. Afterwards, we purified PHYB-YFP-containing complexes using anti-GFP antibody coupled agarose beads and examined whether the immunoprecipitation (IP) altered the ratio of PHYB-bound SUMO-PIF3 to PIF3. Our western blot analysis indicates that the ratio of the SUMO-PIF3 to PIF3 is similar in the 'before the IP' (input) and 'after the IP' lanes indicating that phyB binds to SUMOylated PIF3 and to PIF3(K13R), mutated in the SUMO acceptor site with equal affinity (Fig 5B, Supporting Figure S9B). We also note that the possibility of slight differences in SUMO-PIF3 – phyB binding under different experimental conditions cannot be excluded.

DISCUSSION

Among the posttranslational modifications of PIF3, phosphorylation and ubiquitination was detected and studied extensively so far. It turned out that phosphorylation of PIF3 occurs at numerous amino acid residues and the increased phosphorylated state of the molecule leads to subsequent ubiquitination and degradation of the protein (Ni et al., 2013), similarly to other PIF3 homologues, PIF4 and PIF5. (Lorrain et al., 2008) (Shen et al., 2007). This is one of the earliest key step of switching plant skotomorphogenesis to photomorphogenic development.

Here we report that PIF3 is SUMOylated at the N-terminal part of the molecule and the PIF3 molecule, possessing K13R mutation shows no detectable SUMOylation. These results do not exclude that SUMO could be attached to other amino acids but strongly indicate that the major SUMOylation site of PIF3 is the Lysine 13. We also note that the SUMOylated pool of PIF3 represents only a minor portion of the total PIF3 amount as we could detect PIF3-SUMO only using SUMO-specific antibody and not with the anti-PIF3. Despite the low amount of PIF3-

SUMO in plants, our phenotypic analyses indicated that even this subtle quantity has effect on plant photomorphogenesis. We found, that seedlings expressing PIF3(K13R)-YFP show hyposensitive light responses having longer hypocotyls and smaller cotyledons in light than those of expressing PIF3-YFP (Fig 2A-C). Being PIF3 an overall negative regulator of photomorphogenesis (Leivar et al., 2008), our data indicates that the biological activity of PIF3 is decreased by SUMOylation in light. This conclusion is further confirmed by those experiments in which PIF3 acts as positive regulator of gene induction and protector against photodamage (Fig 2D and Fig 3). Thus taken together, the PIF3(K13R) SUMO acceptor mutant functions as a 'hyper-active' PIF3 possessing increased activity in different responses.

To reveal the mechanistic explanation of this phenomenon we hypothesized that the following properties/functions of PIF3 might be affected by SUMOylation: (i) DNA binding (ii) degradation in light; (iii) intracellular localization; (iv) its complex formation and (v) co-degradation with phyB. The next paragraphs discuss these possibilities.

PIF3 acts as a transcription factor binding to G-box promoter elements (Zhang et al., 2013). We observed that PIF3(K13R) binds to these elements with higher affinity than its WT counterpart in an *in vitro* EMSA assay (Fig 4, Supporting Fig S7-S8). This result is further supported by the light induced gene expression *in planta* which shows that the expression of PIF3(K13R) leads to higher expression levels of early light response genes (Fig 3). These data support the idea that SUMOylation decreases the activity of PIF3, in this regard by interfering with its DNA binding.

We monitored PIF3 degradation in irradiated etiolated seedlings and found that PIF3(K13R)-YFP degrades similarly quickly like its wild type counterpart, PIF3-YFP. We also noticed that both chimeric proteins showed a characteristic upshifted band (Supporting Fig S4) which is the result of protein phosphorylation and was observed earlier (Ni et al., 2013). We also found no difference in the intracellular localization and light-induced nuclear speckles formation of PIF3-YFP and PIF3(K13R)-YFP (Supporting Fig S5). Based on these results, obtained by the available methodology, we can conclude that PIF3 SUMOylation does not alter the light-induced degradation and intracellular localization of PIF3.

Light does not solely initiate the degradation of PIF3 but also destabilizes the PIF3-phyB complex resulting in also the degradation of the photoreceptor (Ni et al., 2014). We found that expressing PIF3(K13R) results in lower amount of detectable phyB compared with PIF3-expressor seedlings (Fig 5A). This data indicates that SUMOylation of PIF3 increases the stability of phyB.

Because phyB-driven responses depend on the available photoreceptor level we postulate that SUMO-regulated PIF3-phyB co-degradation can fine-tune photomorphogenic responses, explaining why lack of PIF3 SUMOylation leads to hyposensitive responses. The SUMO acceptor site K13 locates close to the APB (Active phyB Binding) motif, which is responsible for the phyB binding (Khanna et al., 2004). It is tempting to speculate that attachment of the bulky SUMO peptide in the proximity of this motif interferes with PIF3-phyB binding. Interestingly, we found that PIF3 SUMOylation does not alter the binding affinity of PIF3 to phyB Pfr (Fig 5B Supporting Fig S9B), indicating that PIF3 SUMOylation modifies phyB levels not simply via PIF3 binding. These observations resemble the results published by Ni et al., (Ni et al., 2013) who found that phosphorylation does not alter the binding affinity of PIF3 to phyB, thus it seems that the mode of action of these PTMs is not the modification of the PIF3-phyB complex stability.

Our data demonstrate that SUMOylation decreases the activity of PIF3 in light signalling. It was shown previously that phyB is also a SUMO target and that SUMOylated phyB mediates impaired light signalling (Sadanandom et al., 2015). It is interesting to note how SUMOylation has opposite overall effects on signalling by decreasing the activity of different components with opposite impact. In light, phyB photoreceptor propagates photomorphogenesis, thus SUMOylation of this positive factor slightly decreases its activity. On the other hand, PIF3 is a negative component of light-dependent development, its SUMOylation therefore results in loss of activity thus more pronounced photomorphogenesis. We also notice that SUMOylation level of the phyB pool is higher during the light, whereas of PIF3 is higher during the dark phase of the day. It is tempting to speculate that SUMOylated PIF3 could modulate phyB stability during the night and in cooperation with phyB phosphorylation -which modulates the thermal relaxation of the receptor (Medzihradszky et al., 2013)- they could set the levels of available phyB Pfr by different mechanisms.

Although all the fine details of PIF3 SUMOylation and its consequences are not known, we note its interesting signalling/regulatory aspects. First, every earlier report showed, that PIF3 signalling is regulated by changing the levels of the available PIF3 protein. Sophisticated and very effective molecular mechanisms can reduce PIF3 levels quickly and drastically after the onset of light. Different kinases phosphorylate PIF3 under different conditions, but its consequence is ubiquitination and degradation of the protein (Ling et al., 2017; Ni et al., 2017). SUMOylation however, changes PIF3 activity but does not alter PIF3 stability drastically in light. In this respect, SUMOylation of COP1 is similar as SUMOylation modifies (increases) COP1 activity (Lin et al.,

2016). Second, the complex formation of phyB with PIFs is a key step in the initiation of photomorphogenic signalling. Although SUMOylation of PIF3 does not alter its binding affinity to phyB, but SUMOylation of phyB could modify the PIF3-phyB complex stability, similarly to PIF5-phyB (Sadanandom et al., 2015). Balancing the SUMOylation of both proteins offers a possibility of further regulation of the complex dynamics. Third, PIF3 does not only play role in light signalling but also in various hormonal signalling pathways, and it is tempting to speculate that SUMOylation can fine-tune them. Fourth, we propose that not only PIF3 but also its bHLH transcription factor homologues are also potential targets of SUMOylation, predicting further and even more complicated regulatory aspects of hormonal, thermal and light signalling. These possibilities could be exciting new issues to be examined in the future.

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AUTHOR CONTRIBUTION

P.B., A.H., A.P-S., E-M.J., É.Á. and A.V performed the experiments; F.N., L.K-B. and A.V. supervised the experiments; F.N., L.K-B. and A.V., designed the experiments and analysed the data; A.V. wrote the article with contributions of all the authors.

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FIGURE LEGENDS

Figure 1. PIF3 is SUMOylated *in planta*.

(A) Alignment of the N-terminal part of PIF3 protein sequences from different species. Numbers indicate the corresponding amino acid position of each protein sequence. The Lys13 amino acid in the Arabidopsis PIF3 (red) was identified by the GPS-SUMO online tool as a SUMO attachment site. Lysine amino acids at the N-terminal part of the PIF3 homologues from different plant

species were also identified as SUMOylation site (bold) by GPS-SUMO. Underlined sequences were found as consensus (ψ–K–X–E/D) SUMOylation sites, whereas the others identified as non-consensus ones.

(B) Arabidopsis *pif3* seedlings expressing the *35S:PIF3-YFP* or the *35S:PIF3(K13R)-YFP* transgene were grown on MS medium in 8 h WL (100 μmol m^{-2} s^{-1}) /16 h dark cycles or in darkness for 7 days (dark). Samples were harvested at the end of the light phase (EOD) and at the end of the night (EON). PIF3-YFP and PIF3(K13R)-YFP were immunoprecipitated by using GFP-Trap agarose beads. Western blotting was used to analyse samples containing identical amounts of fusion proteins (loading control, lower panel). PIF3-YFP and PIF3(K13R-YFP) were detected by anti-GFP, whereas Arabidopsis SUMO1 conjugated PIF3-YFP was visualized using anti-SUMO1 antibody. Numbers below the immunoblot image show the relative amount of detected SUMO-PIF3-YFP signal normalized to the dark-grown sample (100%).

Figure 2. SUMOylation of PIF3 results in reduced biological activity in light.

(A) Hypocotyl length of Arabidopsis seedlings grown at different fluence rates of red light was determined after 4 days of growth and was normalized to the corresponding dark values. Error bars indicate standard error. Examined lines: Col: Columbia wild type; *phyB-9*: mutant lacks functional phyB; *pif3*: mutant lacking functional PIF3; transgenic lines in *pif3* background: PIF3: *p35S:PIF3-YFP*; PIF3(K13R): *p35S:PIF3(K13R)-YFP*; hPIF3: *p35S:PIF3-YFP* expressed at high level; hPIF3(K13R): *p35S:PIF3(K13R)-YFP* expressed at high level. Asterisks indicate significant differences between the PIF3 and PIF3(K13R) lines, calculated by the Student's t-test: ***P < 0.005.

(B) Hypocotyl length of *pif3* seedlings expressing the *35S:PIF3-YFP* or the *35S:PIF3(K13R)-YFP* transgene were grown on MS medium in 8 h WL (100 μmol m^{-2} s^{-1}) /16 h dark cycles for 7 days. Error bars indicate standard error. Asterisks indicate significant differences between the marked lines, calculated by the Student's t-test: ***P < 0.005.

(C) Cotyledon area of the seedlings propagated as described in (B).

(D) Survival rate of 4-day-old etiolated Arabidopsis seedlings was calculated after 2 days of white light irradiation (100 μmol m^{-2} s^{-1}). Error bars depict standard error. Asterisks indicate significant differences between the marked lines, calculated by the Student's t-test: **P< 0.05, ***P < 0.005. Line names are indicated as in (A).

Figure 3. PIF3-dependent induction of early response genes.
5-day-old etiolated Arabidopsis seedlings (black columns) were irradiated with 10 μmol m^{-2} s^{-1} R light for 1 h (red columns) and the levels of *ELIP2* (**A**) or *CHS* (**B**) transcripts were measured by qRT-PCR. The mRNA levels were normalized to *TUBULIN (TUB)* levels. Error bars show standard error calculated from 3 independent experiments. Asterisks indicate significant difference (Student's t-test, ***P<0.005).

Figure 4. Electromobility shift assay show stronger DNA binding of PIF3(K13R) than PIF3.
Equal amount of biotin-labelled double-stranded probes were incubated with Arabidopsis PIF3 or PIF3(K13R) expressed and purified from *E. coli* which also expressed Arabidopsis SUMO activation and conjugation enzymes together with SUMO3. The amount of PIF3 and PIF3(K13R) proteins used in the binding reaction is indicated at the top of the image. Binding reactions were resolved on 6% native polyacrylamide gels. The 26-nuclotide-long probe represented a fragment of the *PIL1* promoter carrying a single G-box element at the centre (*PIL1a* probe from (Zhang et al., 2013)). PIF3-DNA and PIF3(K13R)-DNA complex are indicated by an arrow, whereas free (non-bound) probes are indicated by an asterisk. Independent repetition experiment is presented in Supporting Figure S7C.

Figure 5. SUMOylation of PIF3 stabilizes phyB but did not affect PIF3-PHYB binding.
(A) 8-day-old etiolated Arabidopsis seedlings expressing PIF3-YFP or PIF3(K13R)-YFP were irradiated for 72 hours with R light (30 μmol m^{-2} s^{-1}) and the level of PHYB was monitored by western blot hybridization using anti-PHYB antibody. The experiment was repeated 4 times. Error bars indicate standard error, asterisks denote the level of significance calculated by the Student's t-test, **P<0.05.
(B) HIS-tagged Arabidopsis PIF3 was expressed in *E. coli* cells, containing plasmids for expressing the Arabidopsis SUMO-activating (E1) and conjugating (E2) enzymes and SUMO3. We affinity purified PIF3 using Ni-NTA Agarose beads and mixed with total plant protein extract, made from *35S:PHYB-YFP/pifq* transgenic (lane 1 and lane 2) or from Columbia seedlings (negative control, lane 3). After protein complex purification using anti-GFP-bound agarose beads (IP, lane 2 and lane 3), proteins were subjected to western blot analysis using anti-HIS antibody.

Lane 1 shows the input without IP. Empty arrow points at protein bands, corresponding to SUMOylated PIF3, whereas filled arrow mark non-SUMOylated PIF3. Numbers below the immunoblot image show the detected SUMO-PIF3 signal as the percentage of the corresponding band representing the non-SUMOylated PIF3 in the same sample.

SUPPORTING MATERIALS

Additional supporting information may be found in the online version of this article

Supporting Figure S1. PIF3 is SUMOylated *in vitro*.
Supporting Figure S2. Detection of SUMOylated K13 in PIF3.
Supporting Figure S3. PIF3 SUMOylation, PIF3-YFP and PIF3(K13R)-YFP transgene levels in the *pif3* mutant.
Supporting Figure S4 Degradation of PIF3-YFP and PIF3(K13R)-YFP in R light.
Supporting Figure S5. Intracellular localisation of PIF3(K13R) resembles of the wild type.
Supporting Figure S6. The K13R mutation does not alter the PIF3 binding to phyB or to the promoter sequence.
Supporting Figure S7. Protein amount determination for the EMSAs and independent repetition for the EMSA using the *PIL1* probe.
Supporting Figure S8. Electrophoretic mobility shift assays show higher DNA binding affinity of PIF3(K13R) than SUMOylated PIF3 to *ELIP2* and *CHS* promoter elements.
Supporting Figure S9. Western blots used for phyB degradation and repetition of the phyB-PIF3 binding assay
Supporting Table S1. Oligonucleotides used in the study.
Supporting Methods S1. Methods used for mass spectrometry and yeast two-hybrid assays.

A

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Arabidopsis thaliana        6 lfrltkaklesaqdrnp
Eutrema salsugineum         6 lfrltkaklesaqdknp
Capsella rubella            6 lfrlanaklesaqdnnp
Aquilegia coerulea          6 lhrmakeklessthqrm
Arachis duranensis          6 lyrlsreklde eingtr
Cucumis sativus             6 lyrvargkldstqdkns
Eucalyptus grandis          6 lfkmakakldsslekpt
Populus trichocarpa         7 lyrmakgkidfsqekdp
Gossypium barbadense        6 lyrmargkldssqdknp
Vitis vinifera              6 lyrmakgkpesvqqk--
Citrus clementina           6 lyqmtkeklds akqknp
Cephalotus follicularis     6 lyrmakgkldsspekdt
Physcomitrella patens      24 fhgpangkldflrskst
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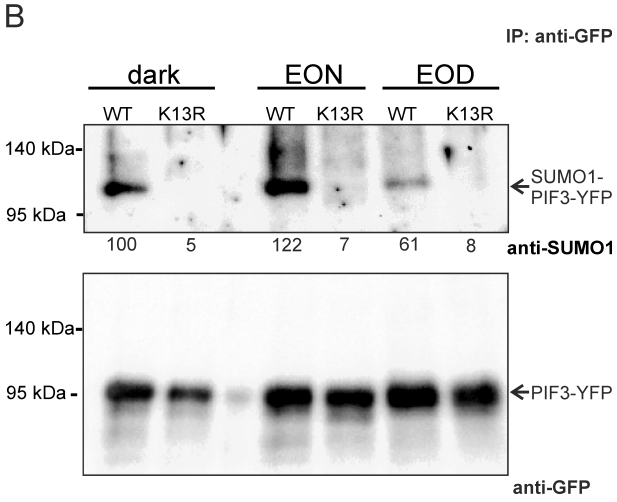
B



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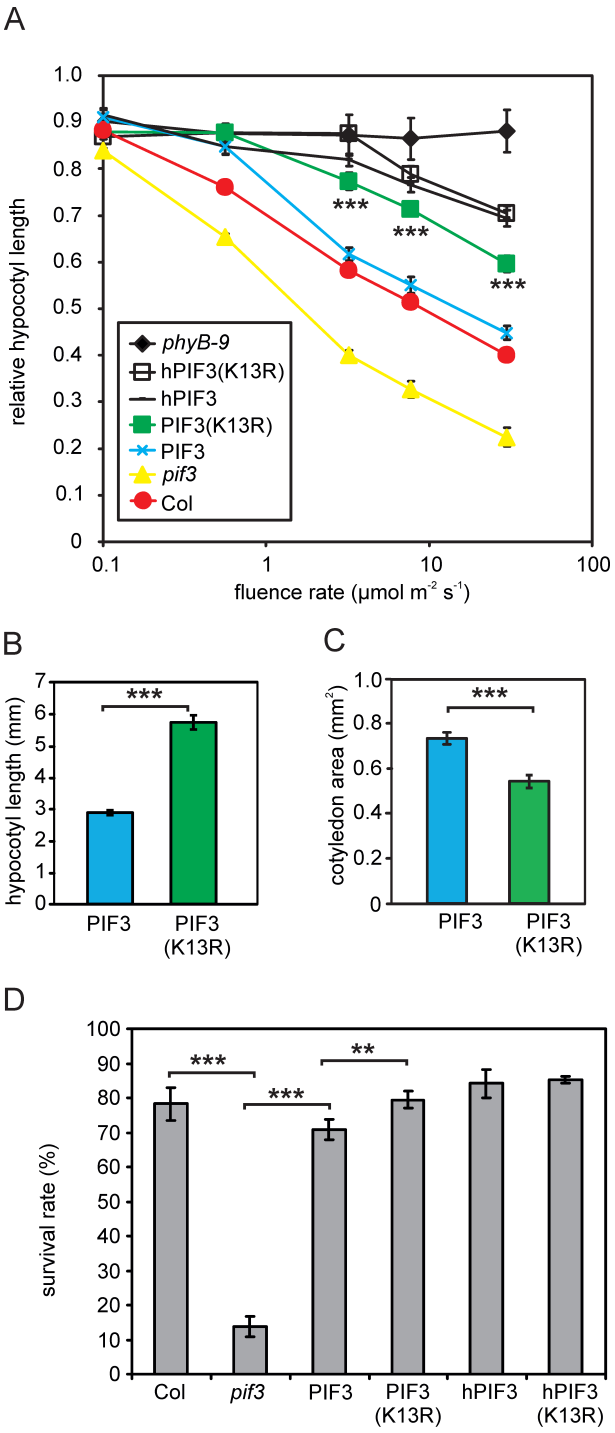

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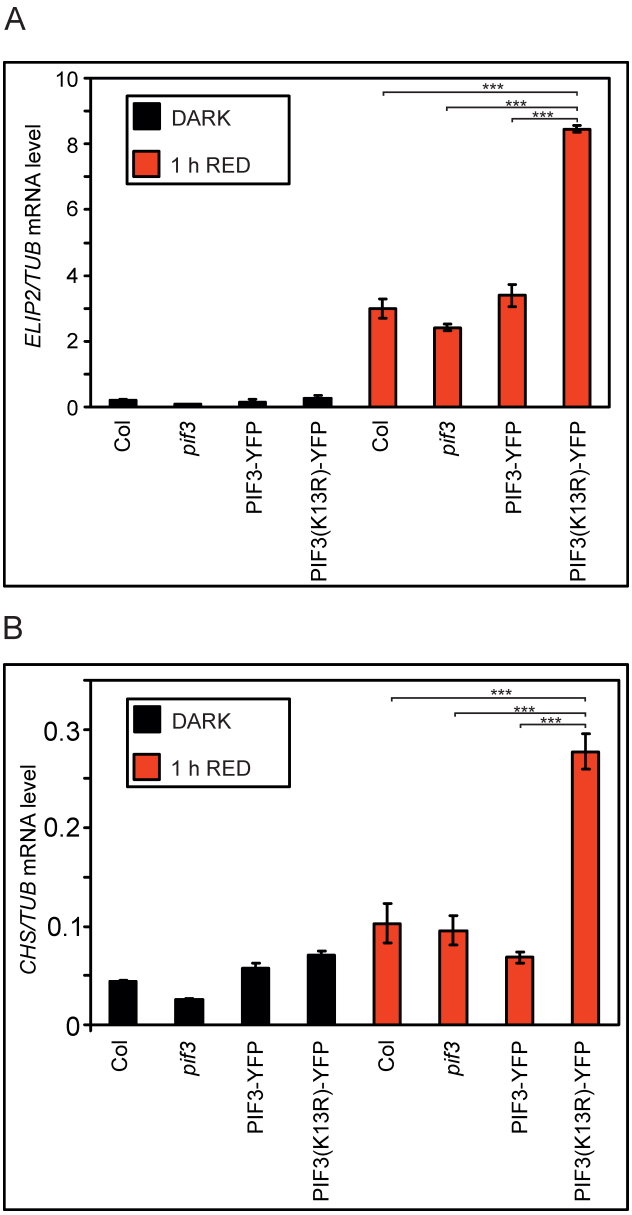

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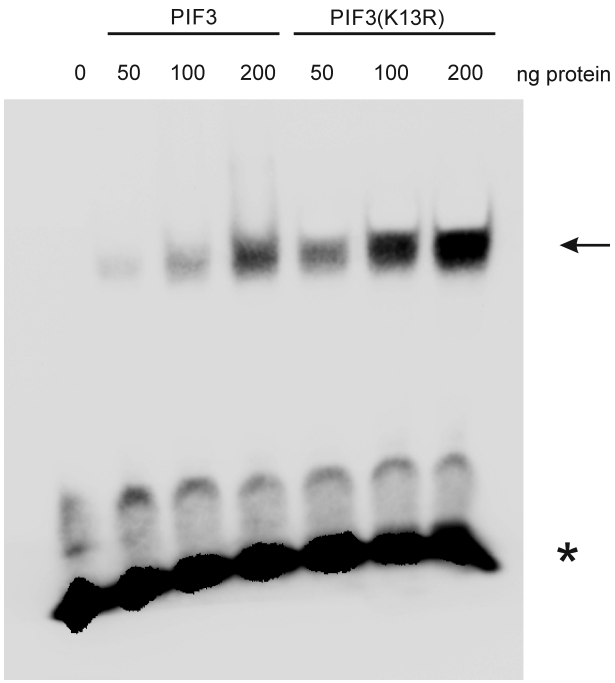

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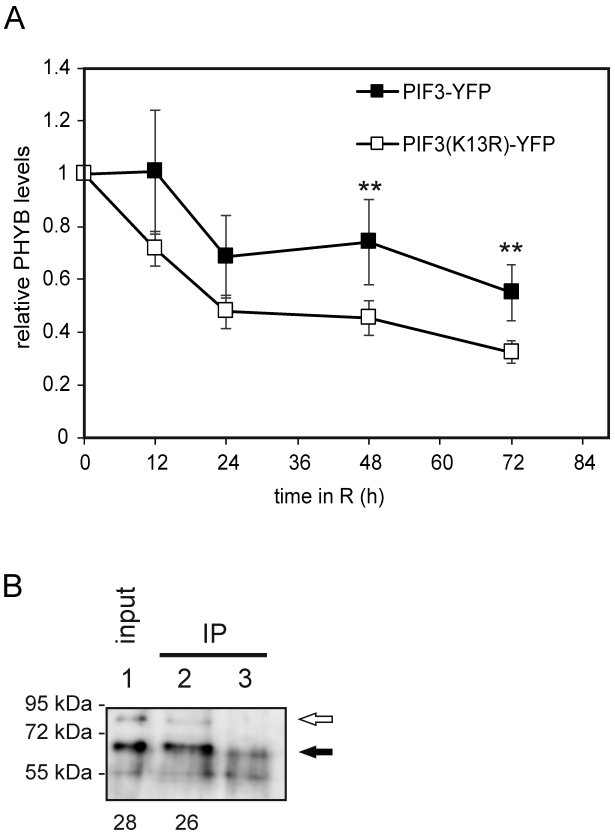

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