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Report

Negative Regulation of PAMP-Triggered Immunity by an E3 Ubiquitin Ligase Triplet in *Arabidopsis*

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Summary

The first line of active defense in plants is triggered by invariant microbial epitopes known as pathogen-associated molecular patterns (PAMPs). Perception of PAMPs by receptors activates a plethora of reactions ending in PAMP-triggered immunity (PTI), which contributes to broad-spectrum resistance [1, 2]. Here, we report a homologous triplet of U-box type E3 ubiquitin ligases (PUBs), PUB22, PUB23, and PUB24 in *Arabidopsis*, that act as negative regulators of PTI in response to several distinct PAMPs. Expression of *PUB22/PUB23/PUB24* was induced by PAMPs and infection by pathogens. The *pub22/pub23/pub24* triple mutant displayed derepression and impaired downregulation of responses triggered by PAMPs. Immune responses including the oxidative burst, the MPK3 activity, and transcriptional activation of marker genes were increased and/or prolonged. Enhanced activation of PTI responses also resulted in increased resistance against bacterial and oomycete pathogens, which was accompanied by increased production of reactive oxygen species and cell death. Our data provide novel insights into the regulation of immunity in plants and links ubiquitination as a mechanism of negative regulation of PTI.

Results and Discussion

We identified three *p*lant *U-b*ox E3 ubiquitin ligases (PUBs), PUB22, PUB23, and PUB24 [3], that are closely related to the tobacco CMPG1, a positive regulator of disease resistance [4] (Figure 1A and Figure S1 available online). The *PUB22/PUB23/PUB24* triplet is strongly and rapidly induced after treatment with the PAMP flg22 (Figure 1B), a conserved epitope of bacterial flagellin [5]. The induction pattern is in fact very similar to that of their close homologs, *PUB20* and *PUB21* [6]. We also observed that the triplet was upregulated upon infection by bacterial and oomycete pathogens (Figures 1C and 1D). To gain insight into the spatial expression pattern, we generated promoter GUS fusions. Four independent transgenic lines for each construct were analyzed. Treatment with flg22 induced the expression of *PUB22* and *PUB23* promoter fusions in mesophyll tissue, whereas expression in control seedlings was mostly limited to the vascular tissue (Figure 1E). In the case of *PUB24*, expression in seedlings was generally lower and induction was stronger in vascular tissue. However, weak activity was also observed in the mesophyll cells. Transgenic plants inoculated with the oomycete *Hyaloperonospora Aarabidopsis* (*Ha*, formerly known as *H. parasitica*) displayed promoter induction, which was strictly limited to penetrated cells with a haustorium (feeding organ, Figure 1E). These data suggest that the PUB triplet may be involved in early immune responses to various pathogens.

To investigate the role of *PUB22*, *PUB23*, and *PUB24* in the immune response, we obtained T-DNA lines all containing insertions in exons (SALK_07261, SALK_133841, and SALK_041046 respectively, Figure S2A) in Col-8 background. The high similarity between *PUB22*, *PUB23*, and *PUB24* suggested some degree of functional redundancy. Therefore, we crossed these lines to obtain double mutants and used the *pub23/pub24* double mutant to cross it with *pub22* and obtain the triple mutant. No transcript was detected for any of the genes, as shown for the *pub22/pub23/pub24* triple mutant (Figure S2C). Unlike *spl11* in rice, which has a nonfunctional U-box [7], we did not observe constitutive induction of PTI or defense-marker genes including *PR1* and *PDF1.2* (Figures S2D and S3) nor any apparent morphological phenotype (Figure S2B). Furthermore, triple mutants were as fertile as the wild-type (WT) Col-8, suggesting that these genes are not essential for plant growth and development.

One of the first reactions triggered by the perception of PAMPs is the oxidative burst, a rapid and transient accumulation of reactive oxygen species (ROS) [5]. We reasoned that if the triplet is required for early immune responses to pathogens, the oxidative burst might be impaired in the mutant. Thus, we measured the oxidative burst triggered by the perception of flg22 in a luminol-based assay. To our surprise, we found that consecutive mutation of *PUB22*, *PUB23*, and *PUB24* lead to a distinct enhancement of the burst compared to the WT (Figure 2A). The increase of the oxidative burst (RLU max) was more pronounced for *pub22/pub23/pub24* ($p > 0.05$) and two double mutants (Figure 2B). Importantly, the downregulation of the production of ROS was delayed (Figures 2A and 2C). We inferred the total amount of produced ROS by integration (RLU total, Figure 2B). Total RLUs, which are proportional to the amount of produced ROS, were significantly higher ($p > 0.01$) for the triple mutant and the three double mutants. Notably, the enhanced oxidative burst was also observed after treatment with the PAMPs chitin $(GlcNac)_8$ [8, 9], a component of fungal cell wall (Figure 2C) and the conserved epitope elf18 from the bacterial transcription factor EF-TU [10] (data not shown). Therefore, the *PUB22/PUB23/PUB24* triplet acts in concert to downregulate the amplitude and duration of the oxidative burst triggered by different PAMPs.

Signaling cascades activated by the perception of PAMPs include a convergent module of mitogen-activated protein kinases (MAPKs). This module leads to the activation of the MAPKs, MPK3, MPK4, and MPK6 [11, 12]. As expected, treatment with flg22 led to the activation of MPK3, MPK4, and MPK6 10 min after treatment with flg22 in the WT (Figure 2D). By contrast, the *pub22/pub23/pub24* triple mutant showed a prolonged activation of MPK3, whereas the activity

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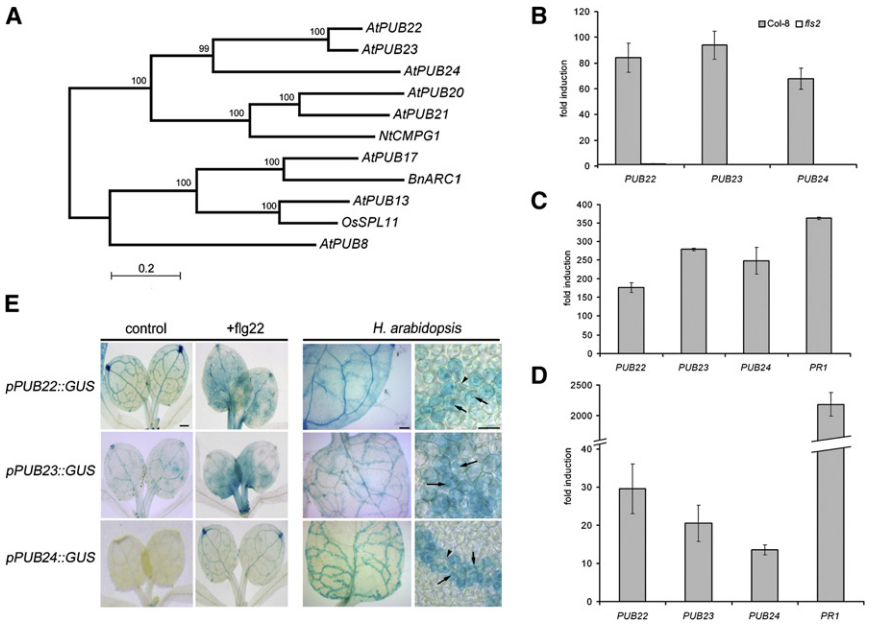

Figure 1. Phylogenetic Relations between U-Box Proteins and Induction of the *PUB22*/*PUB23*/*PUB24* Triplet by PAMPs and Pathogens

(A) Phylogenetic relations between U-box proteins. Phylogeny was calculated with ClustalW2, and the phylogenetic tree was generated from the unmodified alignment with MEGA4 software.

(B) *PUB22*, *PUB23*, and *PUB24* are induced upon treatment with flg22. We treated 14-day-old *Arabidopsis* Col-8 and *fls2* mutant seedlings with 10 μM flg22 or water (control) and harvested them 1 hr after treatment.

(C) *PUB22*, *PUB23*, and *PUB24* are induced in response to *Pst*. Col-8 plants were syringe infiltrated with *Pst* at a concentration of 1×10^7 c.f.u./ml or 5 mM $MgCl_2$ (control) and were harvested 1 day after inoculation.

(D) *PUB22*, *PUB23*, and *PUB24* are induced in response to *Ha*. Col-8 seedlings were spray inoculated with *Ha* Emco5 at a concentration of 5×10^4 spores/ml or mock inoculated (control) and harvested after 7 days.

(B–D) *ACT1* was used as a reference gene and *PR1* as a control for defense induction (C and D). Similar results were obtained from two independent experiments, and standard deviation (n = 3) is represented by error bars.

(E) Promoter regions of *PUB22*, *PUB23*, and *PUB24* are activated in response to flg22 and *Ha* Emco5. For flg22 treatment (10 μM), 2-week-old transgenic seedlings in Col-8 background were grown in half MS liquid media and harvested 1 hr after treatment. For *Ha* Emco5 inoculation, 2-week-old seedlings grown on soil were spray inoculated with 5×10^4 spores/ml and harvested after 1 week. Arrowheads indicate *Ha* hyphae, and arrows indicate penetrated mesophyll cells with a haustorium. Scale bars on the first and third columns represent 200 μm, and the scale bar on the fourth column represents 50 μm.

of MPK4 and MPK6 were unaffected. We also determined the amount of each MPK by western blotting and observed that the protein abundance was unchanged. This suggests that MPK3 might not be a direct target of the PUBs for 48-Lys polyubiquitination leading to degradation (Figure 2D), but does not exclude other modes of ubiquitination that could modulate its activity [13].

Reactions after the oxidative burst and the activation of the MAPK signaling cascade also include transcriptional induction [6]. We therefore analyzed a set of PTI marker genes and observed that *OXI1* [14] and *WRKY29* [12] were upregulated more strongly in the *pub22*/*pub23*/*pub24* triple mutant (Figure 2E). Genes encoding enzymes involved in the production of ROS such as *RbohD* [15] also showed enhanced transcript accumulation in the triple mutant. By contrast, the transcription levels of *FRK1*, *WRKY22*, or *RbohF* were unaffected (Figure 2E and Figure S3). In addition, the defense-related gene *PR1* showed also enhanced induction in response to the bacterial pathogen *Pseudomonas syringe* pv *tomato* DC3000 (*Pst*, Figure 2F).

The U-box is a modified RING domain that mediates the binding to the conjugating enzyme (E2) during the ubiquitination process [16]. E3 ubiquitin ligases mediate the attachment of ubiquitin moieties and thus are responsible for target specificity [17]. To assess whether PUB22, PUB23, and PUB24 possess E3 ubiquitin ligase activity, we expressed and purified Glutathione S-transferase (GST) fusion proteins and mutant variants in *Escherichia coli*. In the presence of all the essential reaction components, all fusion proteins displayed E3 ubiquitin ligase activity (Figure 3 and Figures S4 and S5). In addition to the typical ubiquitination signal detected by anti-polyubiquitin antibody, a laddering pattern showing the addition of single ubiquitin moieties to the GST-PUB fusions was observed indicating self-ubiquitination activity (Figure 3 and Figures S4 and S5, lower panels). Mutation of conserved cystein and tryptophan amino acids to alanine within the U-box motif abolished the ligase activity (Figure 3 and Figures S4 and S5), indicating that PUB22, PUB23, and PUB24 all have an E3 ubiquitin ligase activity.

To test whether these E3 ubiquitin ligases negatively regulate disease resistance, we analyzed the interaction with bacterial and oomycete pathogens. The bacterial pathogen *Pst* showed full virulence on the WT. Little or no difference was observed in *pub22*, *pub23*, or *pub24* single mutants. However, the *pub22*/*pub24* double mutant and the *pub22*/*pub23*/*pub24* triple mutant showed ~30 times less bacterial growth (Figure 4A). We obtained similar results when plants were inoculated with a *Pst* strain lacking the effectors (virulence factors) *AvrPto* and *AvrPtoB* (Δ*avrPto*/*PtoB*). Absence of these two effectors lead, as expected, to less virulence as reflected by a reduced bacterial growth in WT [18] (Figure S6A). However, the small contribution to resistance of single *PUB* genes became apparent. This also reflects the fact that these effectors are able to suppress PTI to increase virulence [18]. The oxidative burst was also enhanced in the *pub22*/*pub23*/*pub24* triple mutant in response to *Pst* during the first 3 hr of the interaction and remained above that of the WT during the measured period (Figure 4B). In addition, microscopic analysis revealed that the triple mutant and the *pub22*/*pub24* double mutant (data not shown) display enhanced accumulation of H_2O_2 as visualized by 3,3′-diaminobenzidine (DAB) staining 24 hr later (Figure 4C). This indicates that the *PUB* triplet is not only involved in the regulation of the early oxidative burst triggered by PAMPs (Figure 2A) but also regulates ROS production during later stages of the immune response.

We also analyzed the interaction with the biotrophic oomycete *Ha* isolate Emco5. The *pub22*/*pub24* double and the *pub22*/*pub23*/*pub24* triple mutants displayed less formation of sporangiophores, indicative of less growth (Figure 4D and Figure S6B). The resistance of the Columbia ecotype against the *Ha* isolate Emco5 is developmentally regulated [19]. Cotyledons are susceptible, whereas in the first true leaves hyphael

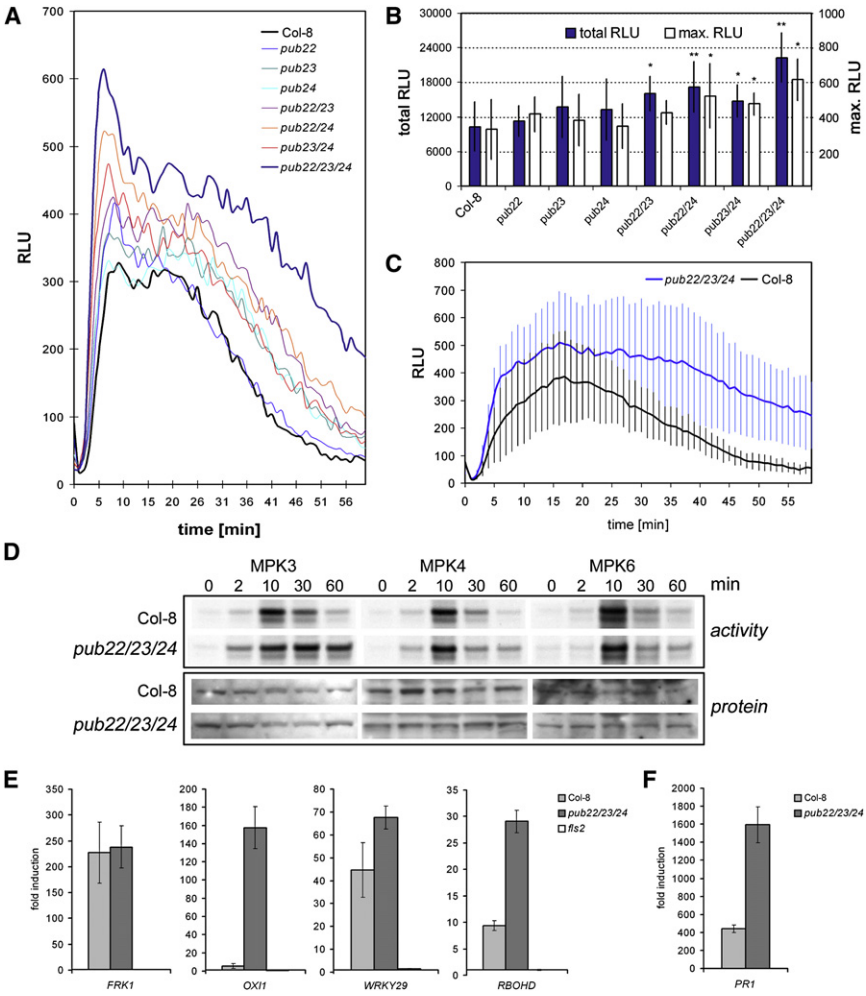

Figure 2. Enhanced PAMP-Triggered Immune Responses in *pub* Mutants

(A) Mutation of *PUB22/PUB23/PUB24* triplet relieves negative regulation of flg22-induced oxidative burst. Oxidative burst was elicited by flg22 (500 nM). Three independent experiments (n = 8) were performed with similar results.

(B) Total and maximal production of ROS after flg22 treatment. The total amount of RLU, which is proportional to the amount of ROS produced, and maximal RLU values (max. RLU of [A]) are shown. Values are significantly enhanced in double and triple *pub* mutants (*$p < 0.05$; **$p < 0.01$). Error bars represent the standard deviation of eight independent samples.

(C) Chitin $(GlcNac)_8$ induced oxidative burst (50 μg/ml) is also enhanced in *pub22/pub23/pub24* triple mutant. The experiment (n = 8) was performed as in (A) and repeated three times with similar results. Error bars represent the standard deviation of eight independent samples.

(D) MPK3 activation by flg22 is prolonged in *pub22/pub23/pub24*. Two-week-old seedlings were elicited with 100 nM flg22, and an immunocomplex kinase assay was performed for MPK3, MPK4, and MPK6. Same extracts were blotted and detected with MPK3-, MPK4-, and MPK6-specific antibodies. Similar results were observed in three independent experiments.

(E) Increased upregulation of PTI and ROS-generation marker genes in *pub22/pub23/pub24*. We harvested 14-day-old seedlings 1 hr after treatment with 10 μM flg22 or water (control).

(F) *PR1* expression is increased in *pub22/pub23/pub24* in response to *Pst*. Plants were syringe infiltrated with *Pst* at a concentration of 1×10^7 c.f.u./ml or 5 mM $MgCl_2$ (control) and harvested 1 day after inoculation.

(E and F) *ACT1* was used as a reference gene. Similar results were obtained from two independent experiments, and standard deviation (n = 3) is represented by error bars.

growth is strongly reduced. This increase in resistance is believed to be due to the activation of effector-triggered immunity (ETI). Microscopical analysis revealed that cotyledons and first leaves displayed enhanced H_2O_2 production (Figure 4E). Small but significant differences in cell death were observed between the *pub22/pub23/pub24* triple mutant and the WT in cotyledons. By contrast, in the first leaves of the triple mutant, cell death was distinctly increased. Taken together, our results show that the *PUB22/PUB23/PUB24* triplet is a negative regulator of immunity during the interaction with the pathogens *Pst* and *Ha*.

ETI is activated by direct or indirect recognition of effector molecules and is characterized by an effective hypersensitive cell death response [1]. Shen and colleagues were able to draw a mechanistic connection between PTI and ETI by linking an ETI-type immune receptor Mla and WRKY transcriptional factors that are PTI repressors [20]. In this sense, the same mechanistic principle could be responsible for the increased cell death observed in the *pub22/pub23/pub24* triple mutant (Figure 4E). Additionally, although previously described U-box and SCF-type E3 ligases CMPG1, PUB17, and ACIF1 [4, 21, 22] were shown to be required for the activation of ETI and cell death, they might modulate related but opposite processes and contribute to PTI activation. Underpinning the regulatory importance of the proteasome for immunity are the different examples of sequestration or manipulation by pathogens of the 26S proteasome-ubiquitin system via effectors either by targeting host proteins for degradation [23, 24] or stabilizing them by its inhibition [25].

The transient nature of PAMP-triggered immune responses is well known, but the underlying molecular mechanisms of downregulation are not understood. Increased and prolonged induction of the oxidative burst was induced by multiple PAMPs (flg22, chitin, and elf18) in the *pub* triple mutant. In addition, examination of public microarray data revealed that the *PUB* triplet is also induced by these PAMPs [6, 9, 26]. Thus, the steady state of the PUB22/PUB23/PUB24 triplet appears to act in concert at the early stage to regulate the amplitude of the immediate PAMP-triggered output and is induced to downregulate responses in later stages (Figures 2A, 4B, and 4E). Notably, meta-analysis of gene expression (http://www.atted.bio.titech.ac.jp/) showed that *PUB22* and *PUB23* can be grouped together with *WRKY22* and *WRKY11* as well as the closely related *PUB20* (*AtCMPG1*) in one regulon. However, *PUB24* shows a divergent regulation suggesting that, although the *PUB22/PUB23/PUB24* triplet acts in concert, they might modulate distinct processes.

Because E3 ubiquitin ligases are substrate determinant factors for ubiquitination, it is probable that the triplet controls the output of one or more targets that are positive regulators of immune responses. This could be achieved either by targeting them for degradation or regulating their activity by

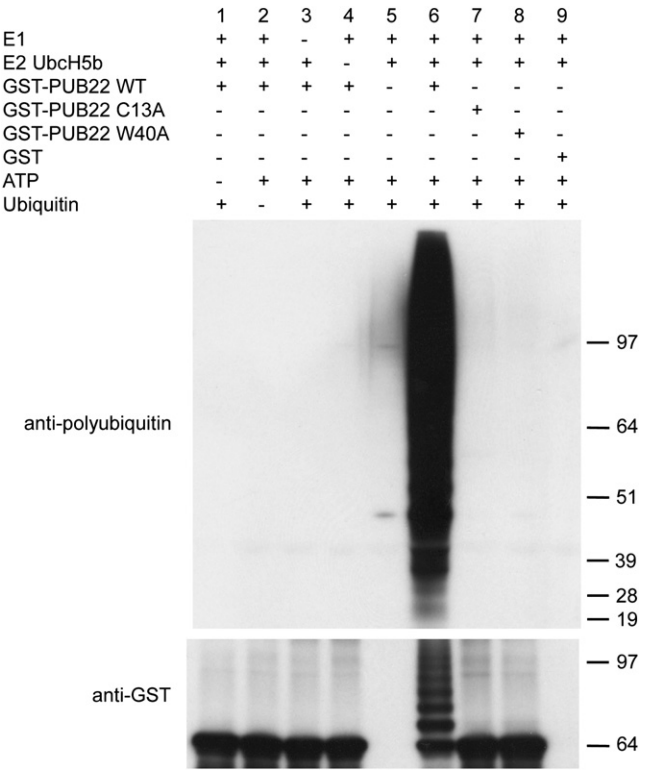

Figure 3. PUB22 Is an E3 Ubiquitin Ligases and Mutation of Conserved Amino Acids in the U-Box Domain Abolishes Its Activity

GST-PUB22 and mutant variants GST-PUB22^{C13A} and GST-PUB22^{W40A} proteins were expressed and purified from *E. coli* and tested for E3 ubiquitin ligase activity with yeast E1 and UbcH5b E2. Lines 1–5 and 9 are negative controls. The reactions were analyzed by protein gel blots with anti-polyubiquitin antibody (upper panel) and anti-GST antibody (lower panel).

noncanonical ubiquitination. In this scenario, an increase of target ubiquitination upon PAMP perception would lead to the reduction of signaling. Because the reduced downregulation of immune signaling is apparent but not complete in the *pub22/pub23/pub24* triple mutant, other uncharacterized factors should also participate in this process.

The negative regulation of signaling cascades by ubiquitination has been described in mammals [13, 27]. Receptor-like kinases (RLKs) such as FLS2 are structurally related to the mammalian receptor tyrosine kinases (RTKs), and downregulation of ligand-induced signaling by endocytosis and relocalization is dependent on multiple monoubiquitination [27]. Binding of flg22 to FLS2 leads to its endocytosis [28], and similar processes have been described for other receptors, for example, BRI1 [29]. Hence, it is conceivable that the involvement of ubiquitin ligases in this process is also conserved in plants. Although ubiquitin ligases such as c-Cbl that regulate endocytosis in mammals by monoubiquitination are not related to the PUB E3s [27], this type of ubiquitination has been shown to be defined in many cases by the interacting E2-E3 complex [30]. Hence, plants may have evolved independent sets of components for signaling, trafficking, and also endosomal structures, which are yet to be characterized. In line with this notion ARC1, a U-box type ubiquitin ligase interacts with the receptor kinase SRK [31]. However, unlike the PUB triplet, ARC1 appears to be a positive regulator of signaling. Whether SRK is ubiquitinated by ARC1 is currently not known. Future challenges will include the identification of ubiquitination target(s) and the mechanisms underlying the negative regulation of PTI by the *PUB22/PUB23/PUB24* triplet.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/18/1396/DC1/.

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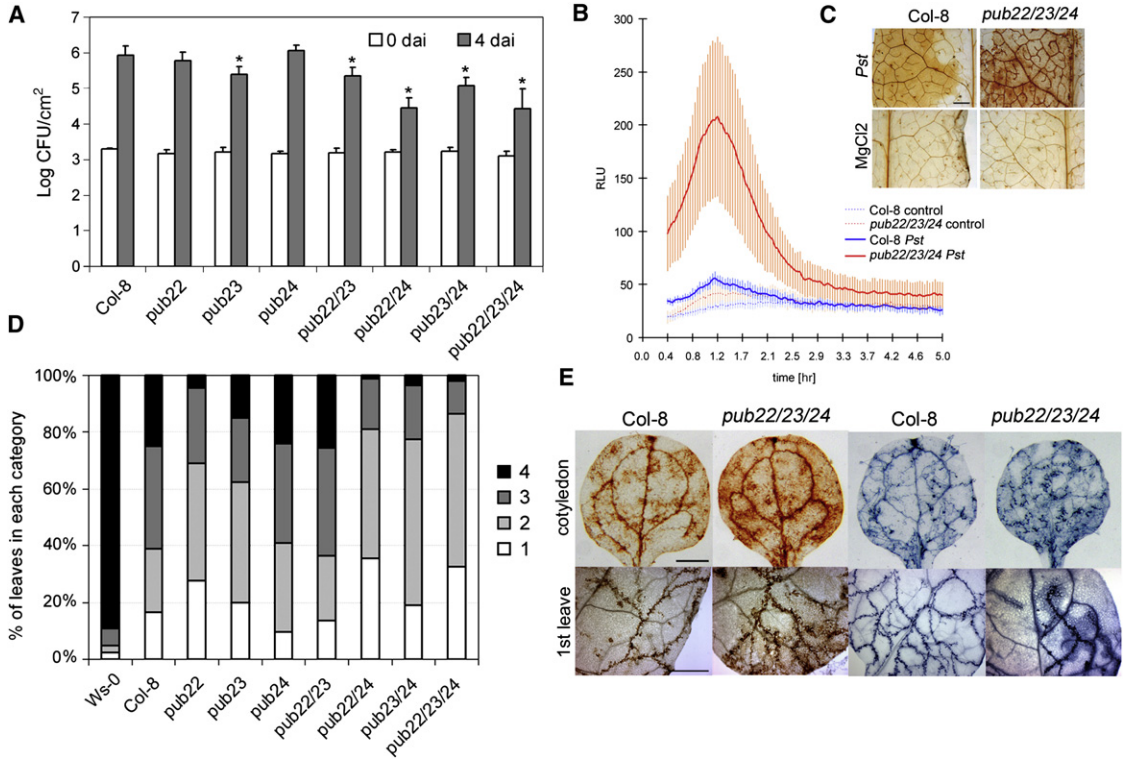

Figure 4. The *PUB22/PUB23/PUB24* Triplet Negatively Regulates Immunity

(A) *pub* mutants are more resistant to the bacterial pathogen *Pst*. Growth of *Pst* on WT *Col-8* and *pub* mutant plants was assessed by spray inoculation of a bacterial suspension of 5×10^8 c.f.u./ml. Bacterial growth was determined at 0 and 4 days after inoculation (dai). Values that significantly decreased in comparison to WT are marked with an asterisk (*$p < 0.05$). Error bars represent the standard deviation from three independent samples, and similar results were obtained in three independent experiments.

(B) Increased resistance is accompanied by an enhanced production of ROS. Leaf discs were inoculated with a solution of 1×10^8 c.f.u./ml *Pst* or mock inoculated (control). Three independent experiments (n = 12) were performed with similar results. Error bars represent the standard deviation of 12 independent samples.

(C) Pressure infiltrated plants with a 1×10^7 c.f.u./ml solution of *Pst* and stained with DAB at 1 dai. The scale bar represents 2 mm.

(D) The *pub* mutants are more resistant to the oomycete pathogen *Ha*. Seedlings were inoculated with a solution of 5×10^4 spores/ml of *Ha* isolate Emco5 and scored 7 days after inoculation on the basis of the number of sporangiophores (0 = 1, 1–10 = 2, 11–20 = 3, >20 = 4) on cotyledons. Bars show the percentage of leaves for each score ($n \geq 40$). The experiment was repeated three times with similar results.

(E) The *pub22/pub23/pub24* triple mutant displays enhanced H_2O_2 production and cell death. H_2O_2 production and cell death were visualized by staining leaves with DAB or Trypan blue respectively in seedlings from (C). Scale bars represent 500 μm.

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