PORPHOBILINOGEN DEAMINASE Deficiency Alters Vegetative and Reproductive Development and Causes Lesions in Arabidopsis

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Abstract

The Arabidopsis rugosa1 (rug1) mutant has irregularly shaped leaves and reduced growth. In the absence of pathogens, leaves of rug1 plants have spontaneous lesions reminiscent of those seen in lesion-mimic mutants; rug1 plants also express cytological and molecular markers associated with defence against pathogens. These rug1 phenotypes are made stronger by dark/light transitions. The rug1 mutant also has delayed flowering time, upregulation of the floral repressor FLOWERING LOCUS C (FLC) and downregulation of the flowering promoters FT and SOC1/AGL20. Vernalization suppresses the late flowering phenotype of rug1 by repressing FLC. Microarray analysis revealed that 280 nuclear genes are differentially expressed between rug1 and wild type; almost a quarter of these genes are involved in plant defence. In rug1, the auxin response is also affected and several auxin-responsive genes are downregulated. We identified the RUG1 gene by map-based cloning and found that it encodes porphobilinogen deaminase (PBGD), also known as hydroxymethylbilane synthase, an enzyme of the tetrapyrrole biosynthesis pathway, which produces chlorophyll, heme, siroheme and phytochromobilin. Our results indicate that Arabidopsis PBGD deficiency impairs the porphyrin pathway and triggers constitutive activation of plant defence mechanisms leading to leaf lesions and affecting vegetative and reproductive development.

Introduction

Lesion-mimic mutants, which spontaneously develop necrotic leaf lesions similar to those caused by pathogen attack, have been identified in Arabidopsis thaliana and other plant species [1,2]. The leaf damage in lesion-mimic mutants resembles the hypersensitive response (HR) that occurs during the plant response to an avirulent pathogen. The HR is triggered by resistance (R) proteins expressed by the host plant; these R proteins recognize specific avirulence (avr) factors expressed by the pathogen. As a consequence of avr recognition by R proteins, a signalling cascade is activated resulting in local cell death and rapid induction of plant resistance genes, finally leading to the activation of systemic acquired resistance (SAR), a broad-spectrum mechanism that confers resistance to further pathogen infection [3,4]. Some lesion-mimic mutants constitutively express cytological and molecular markers associated with defence against pathogens and activated SAR [5].

Several mutations causing lesion-mimic phenotypes have been cloned and some of these genes encode tetrapyrrole biosynthesis enzymes. For example, in maize necrotic leaf lesions are caused by loss of function of LeL22 (Lesion mimic22) and cf1 (camouflage1), which encode uroporphyrinogen decarboxylase III (UROD) [6] and porphobilinogen deaminase (PBGD); also known as hydroxymethylbilane synthase; [7]), respectively. Also, Arabidopsis LESION INITIATION2 (LIN2) encodes coproporphyrinogen III oxidase (CPO) [8] (Figure 1). Anti-sense-RNA mediated inhibition of genes encoding tetrapyrrole biosynthesis enzymes, such as the Arabidopsis glutamyl-tRNA reductase (GluTR; Figure 1) [9], and protoporphyrinogen IX oxidase (PPO; Figure 1) [10], can also cause lesion mimic phenotypes. Similarly, in Nicotiana tabacum, lesion mimic phenotypes are caused by RNA interference-mediated repression of CPO [11,12], UROD [12,13], PPO [14] and FeCh (ferrochelatase, an enzyme that acts in the heme branch of the tetrapyrrole biosynthesis pathway) [15].

The plastids of higher plants synthesize four classes of tetrapyrroles: chlorophyll, heme, phytochromobilin and siroheme, through a branched pathway whose enzymatic steps are well characterized [16,17,18] (Figure 1). Nearly 2% of Arabidopsis proteins bind tetrapyrroles, which act as cofactors in a number of
fundamental biological processes such as photosynthesis, electron transport, oxygen transport and storage; detoxification, nitrogen fixation and light perception [17]. A role for tetrapyrrole biosynthesis intermediates, such as Mg-protoporphyrin IX, as retrograde signalling molecules transmitting information from the plastids to the nucleus to coordinate the expression of their retrograde signalling molecules transmitting information from the plastids to the nucleus to coordinate the expression of their

Figure 1. The tetrapyrrole biosynthetic pathway in plants. Enzymes (in bold capital letters) and intermediates of the nine steps of the common part of the pathway as well as the four end-products (in italics) are shown. GTS: Glutamyl-tRNA synthase; GluTR: Glutamyl-tRNA reductase; GSA-AT: Glutamate-1-semialdehyde amino-transferase; ALAD: 5-aminolevulinic acid-dehydratase; PBGD: porphobilinogen deaminase; UROS: Uroporphyrinogen III synthase; UROD: Uroporphyrinogen III decarboxylase; CPO: coproporphyrinogen III oxidase; PPO: protoporphyrinogen IX oxidase. Asterisks indicate genes for which a mutant phenotype has been reported in Arabidopsis. Redrawn from [23].

doi:10.1371/journal.pone.0053378.g001
No obvious alterations were found in other organs of rug1, although the mutant plants were of reduced height (Figure 2g).

rug1 is Similar to Lesion-mimic Mutants

The damaged areas of the leaves of lesion-mimic mutants express different cytological and molecular markers associated with the disease resistance response; plants undergoing HR after a pathogen attack also express these markers [5,26,27]. The similar lesion phenotypes of rug1 and lesion-mimic mutants prompted us to investigate if some of these markers were expressed in the chlorotic areas of rug1. For this purpose, we stained rug1 plants and leaves with toluidine blue (TB) to detect cuticle defects [28], diaminobenzidine (DAB) to detect H2O2 accumulation [29] and trypan blue (TP) to detect cell death [30]. TB staining revealed that areas of defective cuticle in rug1 leaves overlap with chlorotic sectors (Figure 4a–c), and TP revealed areas of dead cells corresponding to lesions (Figure 4d–g). DAB treatment also detected H2O2 accumulation in the damaged areas of rug1 leaves (Figure 4h–o); moreover, the sizes of the DAB-stained areas were much higher under 16-h light/8-h dark culture conditions (Figure 4l–o) than under continuous light (Figure 4h–k).

The accumulation of salicylic acid (SA) and the expression of some genes encoding pathogenesis-related proteins (PR) are associated with the formation of necrotic sectors in several lesion-mimic mutants and in wild-type plants infected by pathogens [31,32]. To study whether these markers were also induced in rug1, we examined the expression of PR1, a classic marker for pathogen infection [5]. For that purpose, total RNA was extracted from 3-week-old Ler and rug1 plants, and we found by qRT-PCR that PR1 was 5.7-fold upregulated in the mutant compared to Ler. Accumulation of transcripts of PR1 and of other genes involved in pathogen responses was also detected in our microarray analysis (see below). Given that SA induces PR1 expression, we also measured by qRT-PCR expression of SID2, which encodes isochorismate synthase 1 (ICS1), the key enzyme in SA biosynthesis. We found that SID2 was 1.5-fold overexpressed in rug1 compared to Ler. Taken together, our results show that rug1 plants form lesions that phenocopy the effects of pathogen infection, as in other Arabidopsis lesion-mimic mutants.

rug1 is Late Flowering

We found that rug1 plants flower moderately later than Ler under continuous light (Figure S1a–c). The Arabidopsis MADS-box gene FLOWERING LOCUS C (FLC) is a potent repressor of flowering [33,34]. Consistent with the delayed flowering phenotype, FLC was upregulated in the rug1 mutants (Figure S1d). We also used qRT-PCR to measure the expression of the flowering-promoting genes FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS/AGAMOUS LIKE-20 (SOC1/AGL20), both of which are repressed by FLC. FT and SOC1/AGL20 were downregulated in rug1, consistent with the late flowering phenotype and FLC overexpression detected in this mutant (Figure S1d).
Given that vernalization, the exposure to a long period of cold temperature (1 to 3 months at ~1°C to 10°C), accelerates flowering in many Arabidopsis accessions and late flowering mutants [35], we also tested the vernalization response of rug1 and found that the cold treatment induced Ler and rug1 plants to bolt earlier, suppressing the lateness of the rug1 mutant (Figure S1b, c).

Given that vernalization induces flowering by repressing FLC [35], we measured FLC expression in vernalized rug1 plants and...
sector. (d–g) Trypan blue staining of (d) Ler and (f) rug1 third-node leaves and (e, g) close-up views of the leaves shown in (d) and (f), revealing dead cells in rug1. (h–o) (b, i, n) Rosettes of the genotypes indicated and (i, k, m, o) visualization of H$_2$O$_2$ accumulation by means of DAB staining of (i, k, m) one or (o) all of their leaves. Plants were grown under (a–k) continuous light or (l–o) long day conditions (16-h light/8-h dark). Bars = (a, b, h, j, l) 5 mm, (c, d, f, i, k, m–o) 1 mm, and (e, g) 200 μm.

discovered a 10.8-fold reduction in FLC transcript levels compared to non-vernalized rug1 plants.

**RUG1 Encodes PBGD**

To better understand the function of **RUG1**, we used map-based cloning to identify the **RUG1** locus. The **RUG1** gene had been mapped at a low resolution [36]. Linkage analysis of an F$_2$ mapping population derived from a cross of Col-0 to rug1 (in the Ler genetic background) allowed us to delimit a 54-kb candidate interval encompassing 19 annotated genes. We sequenced the transcription units of several genes within the interval and found a single difference between the **rug1** mutant and the wild-type Ler: a C→T transition at position 1,212 (numbering from the predicted translation initiation codon; Figure 3) of the At5g08280 gene, which encodes porphobilinogen deaminase (PBGD; see below). The sequence change in **rug1** is predicted to cause an Ala→Val substitution in the **RUG1** protein at position 246, a residue that is highly conserved among PBGDs (Figure 5). To confirm that the mutation in At5g08280 causes the phenotype of the **rug1** mutant, we complemented the mutant phenotype of **rug1** with a transgene carrying the **RUG1** wild-type coding sequence fused to the 35S promoter (Figure S2a–c; see Methods).

The **RUG1** open reading frame is predicted to encode a 382 amino acid protein of 41.04 kDa, porphobilinogen deaminase (PBGD; EC 2.5.1.61), which catalyzes the fifth enzymatic step of the tetrapyrrole biosynthesis pathway (Figure 1): the deamination and polymerization of four molecules of porphobilinogen in the linear tetrapyrrole 1-hydroxymethylbilane [37,38]. PBGD has been purified from a wide-range of prokaryotic and eukaryotic organisms [39]. In animals and yeast, PBGD is a cytosolic protein but in higher plants and algae, it is targeted to the chloroplast [40]. In Arabidopsis, PBGD is a chloroplast protein encoded by a single-copy gene [41,42]. The overall sequence similarity between the PBGD of Arabidopsis and other organisms is moderately high: 76, 73, 74, 46, 38, 37, 37 and 35% identity for pea, wheat, rice, *Escherichia coli*, human, mouse, *Daphnia pulex*, and *Saccharomyces cerevisiae*, respectively (Figure 5). This is consistent with the properties of Arabidopsis PBGD, which is very similar to other PBGDs [39].

**RUG1** is broadly expressed, as shown by data deposited at different publicly available microarray databases [Genevestigator (https://www.genevestigator.com/go/) and the BIO-array resource (BAR; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)] and consistent with other experimental results that detected PBGD in different organs of Arabidopsis [41,42] and pea [40]. Interestingly, we found that overexpression of Arabidopsis PBGD in a wild-type genetic background leads to the appearance of supernumerary shoot apical meristems and occasionally small necrotic patches in the leaves (Figure S2d–f).

**Light Affects the Phenotype of rug1**

In maize, defective PBGD function causes the appearance of yellow sectors in the leaves of the *cf1* mutant under light/dark cycles, a phenotype that is suppressed when *cf1* plants are grown under continuous light [7]. Because we normally grow our plants under continuous light, we also tested whether growth under light/dark cycles could modify the *rug1* lesion phenotype. *rug1* plants grown under long-day conditions (16-h light and 8-h dark) displayed an apparent increase in the size of the chlorotic sectors and a reduction of plant growth compared to those grown under continuous light (Figure S3a, b, d, e). Remarkably, when *rug1* plants were grown under 16-h light/0-h dark conditions for 15 days followed by 8 days under continuous light, the lesion sectors were almost completely absent from the leaves (Figure S3c, f). These results indicate that sector formation in *rug1*, as in maize *cf1*, is dependent on the photoperiod conditions.

We also examined whether the lesion phenotype of *rug1* was affected by different light intensities by growing mutant and wild-type plants under light intensities lower (35 μmol m$^{-2}$ s$^{-1}$) and higher (115 μmol m$^{-2}$ s$^{-1}$) than those of our standard culture conditions (usually 65–70 μmol m$^{-2}$ s$^{-1}$). We found that the extent of the necrotic areas of *rug1* leaves were increased and reduced at the higher and lower light intensities, respectively (Figure S4a). We also grew *rug1* seedlings in the dark for 10 days to assess the photomorphogenic response of the mutant, but we observed no differences with Ler (Figure S4b).

**PBGD and Catalase Activities are Reduced in rug1**

To biochemically assess PBGD activity in *rug1*, extracts were obtained at 21 das from mutant and wild-type rosettes of plants grown under 16-h light/8-h dark photoperiod or continuous light conditions. Compared to Ler, we detected a 24% reduction in PBGD activity in *rug1* activity under long day conditions and a 16% reduction under continuous light conditions (Figure 6a). Consistent with the decreased PBGD activity in *rug1*, the substrate of PBGD, porphobilinogen (PBG), accumulated in the mutant to levels significantly higher than in wild-type plants (Figure 6b). PBGD participates in the biosynthesis of heme, a cofactor of ROS scavenging enzymes such as catalase, and a defect in PBGD function in maize *cf1* causes a reduction in catalase activity [7]. To assess if the *rug1* mutation affects catalase, we measured this activity in mutant and wild-type rosettes. We found a moderate reduction in catalase activity in *rug1* plants grown under long day conditions (Figure S5).

**Auxin Response and Photoautotrophic Growth are Altered in rug1**

Since several genes related to auxin signalling were downregulated in *rug1* (Table S1), we investigated whether the auxin response was altered in *rug1*. Root elongation was examined in *rug1* and Ler plants grown on media supplemented with different indole-3-acetic acid (IAA) concentrations. The *rug1* plants had moderately reduced IAA sensitivity, revealing a relationship between porphyrin biosynthesis and auxin responsiveness (Figure S4c).

Given that PBGD participates in chlorophyll biosynthesis and that *rug1* exhibits a reduction in size, we also studied whether photoautotrophic growth was altered in the *rug1* mutants. To this end, *rug1* and Ler plants were grown in culture media with or without 1% sucrose. We found that *rug1* growth was somewhat impaired when sucrose was not present: 12.5% of *rug1* seedlings were found to be developmentally arrested at the stage of green expanded cotyledons and first pair of tiny leaves versus 3.5% in Ler (Figure S4d).
We also used microarray analysis to examine the effect of impaired RUG1 function on the Arabidopsis nuclear genome. We found 280 genes that were significantly misregulated, by at least 1.5-fold, in rug1, 173 (61.8%) upregulated and 107 (38.2%) down-regulated (Table S1). The genes were categorized either as known (233) or unknown (47) based on the annotations at the Arabidopsis Information Resource (TAIR; www.arabidopsis.org). The known genes were further classified into 13 different functional categories mainly based on the Functional Catalogue (FunCat) [43] assignments of the Munich Information Centre for Protein Sequencing (MIPS; http://mips.gsf.de) and literature reports [44] (Table S1). The largest categories identified were: “cell rescue, plant defence, senescence and virulence” (61 genes, 21.8%), “metabolism” (57 genes, 20.3%), “transcription” (28 genes, 10%) and “cellular communication/signal transduction” (23 genes, 8.2%). The main category includes genes encoding proteins involved in plant defence or resistance to pathogens, and most of these genes were overexpressed in rug1 compared with Ler (43 genes, 70.5%), consistent with the rug1 lesion phenotype (Table S1). Thus, we identified proteins belonging to different plant pathogenesis-related (PR) families such as PR1, the plant defensin-defense proteins PDF1.1, PDF1.2a, PDF1.2b, PDF1.3c, PDF1.5 and PDF1.4 (PR-12 family) [45,46,47], the lipid transfer protein 2 (LPT2; PR-14 family) [48], and a chitinase class IV protein (PR-3 family). This category also included the NPR1/NIM1 interacting protein NIMIN1 required for fine-tuning PR1 expression [49], several members of the TIR-NBS family of plant disease resistance proteins (R proteins) [50] and the FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1), whose expression is activated by bacterial flagellin and confers resistance to bacterial and fungal pathogens [51]. Other genes included in this category encoded proteins associated with senescence (SAG13 and SAG101) [52,53], cell death (e.g. the ankyrin domain containing protein ACCELERATED CELL DEATH LIKE2 (ACL2) similar to ACD6, which activates SA-dependent cell death [54], detoxifying enzymes (P450 cytochromes, glutathione S-transferases, peroxidases and a heavy-metal-associated domain protein) or abiotic stress-responsive factors, such as the cold-responsive gene KIN2/COR6.6 [55] and heat-shock factor 4.

In the “cellular communication” FunCat category, several putative signal transduction components were upregulated in rug1, including receptor-like protein kinases, protein kinases, calmodulin and calcium-binding proteins, which might potentially activate genes of the “cell rescue, plant defence, senescence and virulence” group. Within the “transcription” category, the most frequently represented transcription factor family was WRKY, whose members participate in pathogen defence, senescence, trichome development and biosynthesis of secondary metabolites [56]. The At2g16400 and At1g08040 genes, encoding WRKY46 and WRKY40 respectively, which are induced by the pathogen elicitor chitin [57] were up-regulated in rug1. The floral repressor FLC was the gene showing the largest fold-change in rug1,
consistent with our qRT-PCR results and the late flowering phenotype of the mutant.

A total of 13 genes related to auxin response (included in the "systemic interaction with the environment" class) were misregulated in rug1, 12 of them belonging to the SMALL AUXIN-UP RNA1 (SAX1) family of auxin-inducible genes (Table S1), which are rapidly upregulated after auxin exposure [58]. The remaining gene, At5g13370, encoded a putative auxin-responsive GH3-like protein. Whereas all the SAX genes were repressed in rug1, At5g13370 was upregulated.

We used the GOrilla web-based application (see Methods) for gene enrichment analysis in the rug1 mutant. Significant enrichment was only shown when the "biological process" ontology was used but not with the "cell component" or "molecular function" options. The lowest \( P \) and false discovery rates (FDR) \( q \) values (2.58 \( \times 10^{-10} \) and 5.7-fold up-regulated as measured by qRT-PCR, and 5.1- and 4.1-fold up-regulated, respectively, as measured by microarray (Table S1). In addition, qRT-PCR and microarray analyses showed 1.9- and 1.5-fold down-regulation, respectively, for SOC1/AGL20. Also, PDF1.1 (At1g75830) and SAG12 (At2g29350) were upregulated 10.4- and 7.4-fold by qRT-PCR, respectively, and 4.1- and 3.9-fold by microarray analysis.

Discussion

Nearly twenty years ago [41] isolated the Arabidopsis gene encoding PBGD. They found that it was a single copy gene in the Arabidopsis genome and that PBGD was targeted to chloroplasts. The same year, [39] published the purification and biochemical characterization of Arabidopsis PBGD. These authors discovered that Arabidopsis PBGD showed properties very similar to those of other prokaryotic and eukaryotic PBGDs, all of which are highly conserved. Despite the time elapsed, to date no work had been published on Arabidopsis PBGD function based on a mutational approach. Therefore, our study of the rug1 mutant allows us, for the first time, to characterize at a genetic and molecular level the Arabidopsis gene encoding PBGD. Only one previous work described the cloning of a plant gene encoding PBGD from the isolation of a mutant: the maize non-clonal sectoring mutant qf1 [7]. A likely explanation for the paucity of plant mutants affecting genes encoding PBGD is that they are single copy genes acting in a primary metabolic pathway, whose null alleles probably would be lethal. Hence, only hypomorphic alleles could be identified and studied.

In contrast with plants, a large amount of information is currently available about the effects of perturbed PBGD function in mammals, particularly in humans. Deficiency in PBGD produces acute intermittent porphyria (AIP), a severe and common form of the acute porphyrinas, a group of inherited disorders caused by dysfunctions of the heme biosynthetic pathway in humans. AIP is associated with neuropathy attacks, including abdominal pain, vomiting and hypertension [59]. More than 300 mutations affecting human PBGD have been identified (The Human Gene Mutation Database; http://www.hgmd.cf.ac.uk/ac/index.php?gene = HMBS), most of which are missense or nonsense mutations. A PBGD-defective mouse model has been developed that reproduces the neuropathic symptoms of human AIP [60]. Two major hypotheses have been invoked to explain porphyric neuropathy: (a) reduction in the levels of heme, and (b) direct toxicity caused by accumulated porphyrin precursors, including PBG.

rug1 plants spontaneously develop chlorotic leaf lesions in the absence of pathogen attack, resembling the phenotype of lesion-mimic mutants. Like these, rug1 exhibits cytological markers frequently associated with the formation of patches of dead tissues. Thus, the staining in rug1 leaves of dead cells by TP and the detection of \( \text{H}_{2}\text{O}_{2} \) by DAB in sites showing signs of damage before staining indicates that rug1 plants form lesions similar to the HR caused by avirulent pathogens or disease symptoms following pathogen attack [25]. In Arabidopsis, lesion formation (named
phytoporphyrin in plants) [6] and the induction of defence responses caused by the inhibition of the activity of other enzymes of the tetrapyrrole pathway have been reported not only for PBGD but also for CPO [9] and PPO [10]. Interestingly, we discovered that overexpression of PBGD may lead to the formation of supernumerary apical meristems and the appearance of small patches of necrosis. This indicates that unbalanced porphyrin synthesis caused by either defective or enhanced activity of tetrapyrrole enzymes (such as PBGD) can have dramatic effects on plant development.

The phenotype of the Arabidopsis rug1 mutant is similar to that of the maize cfl1 mutant. The similar phenotypic effect caused by defective PBGD in a monocotyledonous and a dicotyledonous species is consistent with the similarity between their amino acid sequences (71.6% identity and 90.6% similarity), biochemical activities and subcellular localization. Along these lines, PBGD activity was reduced in cfl1 and rug1, and both mutants exhibited increased porphobilinogen levels. Nevertheless, the reduction in PBGD activity was higher in cfl1 than in rug1, which is consistent with their molecular lesions, since rug1 carries a missense mutation that affects a highly conserved residue of the RUG1 protein and cfl1 bears a Mutator transposon inserted in its 5′ UTR that strongly diminishes CFI expression [7].

Sectoring is notably enhanced in rug1 plants grown under a light/dark cycle rather than under continuous light. [7] proposed a threshold model to explain the variegated phenotype of the cfl1 mutant of maize. According to this model, defective PBGD results in a reduction of the capacity to scavenge reactive oxygen species (ROS), especially in the bundle sheath cells, since heme is a cofactor of several ROS scavenging enzymes. As a consequence, an increase in cellular damage results in the formation of yellow sectors. The authors argue that lower levels of NADPH and antioxidant pools formed in the dark, together with the decreased ROS scavenging potential of cfl1 bundle sheath cells, would lead to a “burst” of oxidative damage upon illumination and thus trigger cell death. This would explain why yellow sectors form in dark/light cycles. Our experimental results showed that catalase activity is reduced in rug1 plants grown under long day conditions (and hence exhibiting large chlorotic areas) as in cfl1 yellow sectors, supporting the model that reduced antioxidant activity is responsible for the formation of damaged areas. Nevertheless, contrary to cfl1 yellow sectors that do not accumulate H2O2 [61] rug1 leaf lesions do accumulate H2O2. Hence, we cannot rule out the possibility that production of ROS caused by PBG accumulation might also contribute to lesion formation in rug1.

A photoperiod effect on the extent of the lesions in tetrapyrrole mutants has also been described for the Arabidopsis lin2 mutant, which exhibits more severe lesions under long day than under short day conditions [8] and the tigrina (tig) mutant of barley, which accumulates the photosensitizer protochlorophyllide and shows sensitivity to dark/light cycles as do rug1 and cfl1 [62].

Consistent with the lesion formation phenotype of rug1 plants and constitutive activation of pathogenesis response mechanisms, our microarray analysis revealed that almost 300 genes were misregulated in the rug1 mutant. The most abundant category was that of “cell rescue, plant defence, senescence and virulence”, and most genes in this category were over-expressed in the mutant. The SA-induced gene PRI, a marker of SA-dependent signaling [63], displayed the highest level of expression, as we confirmed by qRT-PCR experiments. PRI expression is a molecular marker of cytological damage and lesion-mimic mutants as well as wild-type plants infected by necrogenic pathogens [5,27]. Besides, like rug1, Arabidopsis lin2 plants affected in CPO accumulate PRI transcripts [8]. The increase in expression of PRI and SID2, which is involved in SA biosynthesis, point to an increase of SA activity in the rug1 mutant. This would be expected since it is widely known that the concentration of SA, which is a signal required to elicit SAR, is high in lesion-mimic mutants such as lin2 and increases after pathogen infection. Moreover, a role for SA in controlling flowering time in Arabidopsis has been proposed [64]. Along these lines, rug1 plants flower later than the wild type, which is caused by the overexpression of the floral repressor FLC and the downregulation of the flowering promoting genes FT and SOC1/AGL20. This is in contrast with previous results showing that increased SA levels promote flowering in Arabidopsis by acting as a negative regulator of FLC expression [64]. A possible explanation for this discrepancy is that the end products or intermediates of the tetrapyrrole pathway might be required for SA to promote flowering in Arabidopsis. Accordingly, delayed flowering has also been reported for Arabidopsis antisense transgenic lines disrupted in the PPO tetrapyrrole enzyme, which exhibited high SA levels, accumulation of PRI transcripts and necrosis similar to that of rug1 [10].

A connection between SA and auxin has been described in Arabidopsis and it has been proposed that pathogens can alter host auxin biosynthesis for their own benefit. In response, the host plants would be able to repress auxin signaling during infection by SA signaling [65,66]. Thus, in a comprehensive study carried out to analyze the effects of SA on auxin signaling it was found that SA globally repressed auxin-related genes, thereby inhibiting auxin responses [66]. Interestingly, we found in our microarray analysis that 13 auxin-related genes were misregulated in rug1. Twelve genes encoding auxin-responsive proteins were repressed; some of these genes belong to the SAUR family, whose transcripts rapidly and transiently accumulate after auxin exposure [67]. The function of these genes, however, is largely unknown, likely due to genetic redundancy [68]. Interestingly, four of the SAUR genes were also repressed in wild-type Arabidopsis plants in response to an SA analog [66]. The remaining auxin-related gene was up-regulated and encoded a protein of the GH3 family, some members of which are IAA-amino acid conjugating enzymes [69]. Hence, auxin induction of genes of the GH3 family is assumed to diminish auxin signaling. Consistent with the hypothesis of auxin signaling being reduced in rug1, our root elongation assay indicated that rug1 was more insensitive than the wild type to exogenous IAA.

In summary, a mutation in the PBGD gene of Arabidopsis has been reported for the first time. Our results reveal that, like in humans, perturbation of the tetrapyrrole pathway at the PBGD level severely disrupts cell metabolic homeostasis, leading to cell damage and even cell death, which has severe harmful effects on growth and development. The availability of the rug1 mutant provides a valuable tool for further in vivo investigation on the function of plant PBGDs.

Materials and Methods

Plant Material, Growth Conditions and Growth Assays

Cultures and crosses were performed as described in [70] and [24], respectively. Seeds of the Arabidopsis thaliana (L.) Heynh. wild-type accessions Landsberg erecta (Ler) and Columbia-0 (Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The rug1 mutant was isolated in the Ler background after ethyl methanesulfonate (EMS) mutagenesis and backcrossed twice to Ler [24]. The lin2 seeds were kindly provided by Atsushi Ishikawa. Light-sensitivity, autotrophic growth and photomorphogenic response analysis were performed as previously described [71]. Root growth inhibition by IAA was carried out as described in
were performed as described in [30] and [28], respectively. Confocal imaging was performed as described in [73]. Trypan-blue (from rosettes of the rug1 mutant and the wild-type Ler collected 21 days, as described in [7].

Morphological, Histological and Biochemical Analyses
Whole rosette and single leaf pictures were taken in a Leica MZ6 stereomicroscope. For light microscopy, plant material was fixed with FAA/Triton (1.85% formaldehyde, 45% ethanol, 5% acetic acid and 1% Triton X-100) as described in [71]. 0.5-μm-thick transverse sections of leaves were cut on a microtome (Microm International HM350S), stained with 0.1% toluidine blue and observed using a Leica DMRB microscope equipped with a Nikon DXM1200 digital camera under bright-field illumination. Confocal imaging was performed as described in [73]. Trypan-blue (for cell death) and toluidine-blue (for cuticle defects) staining were performed as described in [30] and [20], respectively. Scanning electron microscopy was carried out as described in [71]. H$_2$O$_2$ was detected by DAB staining as described in [29]. PBG concentration and PBGD and catalase activity were measured from rosettes of the rug1 mutant and the wild-type Ler.

Positional Cloning and Molecular Characterization of the rug1 Mutations
To clone the RUG1 gene, SSLP, SNP and CAPS markers were designed according to the polymorphisms between Landsberg erecta (Ler) and Columbia-0 (Col-0) described in the Monsanto Arabidopsis Polymorphism Collection database (http://www.arabidopsis.org). For allelic sequencing, PCR products spanning the At5g08280 transcription unit were obtained using as a template wild-type and mutant genomic DNA and the oligonucleotide primers shown in Table S2 and Figure 5. Sequencing reactions were carried out with ABI PRISM BigDye Terminator Cycle Sequencing kits in 5-μl reaction volumes. Sequencing electrophoreses were performed on an ABI PRISM 3100 Genetic Analyzer.

Complementation of the rug1 Mutation and RUG1 Overexpression
The coding region of At5g08280 was amplified by PCR using the attB-containing primers shown in Table S2 and a proofreading polymerase (Pfu Ultra; Stratagene). The product was firstly cloned into the pGEM-T Easy221 vector (kindly provided by B. Scheres) and then subcloned into the pMDC32 vector by recombinaction using Gateway technology (Invitrogen). Chemically competent E. coli DH5α cells were heat-shocked and transformants were isolated and confirmed by PCR. Plasmid DNA was obtained and transformed into competent Agrobacterium tumefaciens LBA4404 cells. Positives clones containing the 35S::RUG1 construct were used for in planta transformation of rug1 and wild-type Ler plants [74]. T$_2$ seeds were sown in agar plates supplemented with 40 mg/ml of hygromycin for isolation of transformant plants. We used PCR to verify the presence of the transgene in the transformants.

Quantitative RT-PCR
Total RNA was extracted from 50 to 70 mg of 3-week-old rosettes (Ler and rug1) and DNase I treated using the Qiagen RNeasy Plant Mini Kit, following the manufacturer’s instructions. The RNA was reverse transcribed and subjected to qRT-PCR as described in [71]. Relative quantification of gene expression data was performed using the $2^{-\Delta\Delta C_{T}}$ or comparative $C_{T}$ method [75]. Each reaction was performed in three replicates and levels of expression were normalized by using the $C_{T}$ values obtained for the housekeeping gene G3PDH.

Microarray Analysis
Ler and rug1 3-week-old plants from 6 different sowings (80 to 100 mg per sample) were frozen in liquid N$_2$ and ground by mortar and pestle. Total RNA was extracted as described in [76] and three biological replicates were obtained for each genotype by mixing two original RNA samples. 10 μg of total RNA from each biological replicate was used for microarray hybridization and analysis. In brief, Superaramine Telechem slides containing more than 26,000 spots corresponding to the Arabidopsis oligo set from Qiagen-Operon, obtained from David Galbraith (Arizona University; http://ag.arizona.edu/microarray/), were hybridized by conventional methods with RNA probes labelled with either Cy3 or Cy5 Mono NHS Esters. For the hybridization, equal amounts of dye of each cDNA sample, ranging from 200 to 300 pmol, were mixed with the hybridization buffer containing 50% formamide, 5×SSC, 1% SDS, 5×Denhardt’s. This mixture was boiled for 5 minutes at 95°C and then added to the prehybridized slide. Hybridization took place overnight at 37°C in a hybridization chamber. Arrays were then washed in an orbital shaker for 5 min at 37°C in 0.5×SSC, 0.1% SDS; twice for 5 min at room temperature (RT) with 0.5×SSC, 0.1% SDS; three times with 0.5×SSC at RT, and 5 min with 0.1×SSC at RT. The slides were then spin-dried and scanned in a GenePix 4000B scanner (Axon Instruments) at 10 μm resolution, 100% laser power, and different PMT values to adjust the ratio to 1.0. Microarray images were analyzed using GenePix 5.1 (Axon Instruments) software.

The data were normalized and statistically analyzed using the LIMMA package [77,78]. For local background correction the “normexp” method in LIMMA was used. The resulting log-ratios were print-tip loess normalized for each array. A multiple testing correction based on the false discovery rate (FDR) was performed to correct p-values. Genes were considered to be differentially expressed if the corrected p-values were <0.05 and their fold change greater than 1.5 fold or lower than −1.5 fold.

For gene enrichment analysis the GORilla web-based application [79,80] (http://ceb-gorilla.cs.technion.ac.il/) was used. Genes were classified into functional categories and visualized choosing two unranked (target and background) lists of genes as running mode. The background list was composed by all the genes on the array. P-values of 10$^{-3}$ and 10$^{-5}$ were selected as thresholds and the results obtained choosing three different ontologies (biological process, cell component and molecular function) were compared. The GORilla tool transformed p-values into FDR q-values using the method described in [81].

Supporting Information
Figure S1 Flowering time in rug1. (a) Ler and rug1 plants, pictured 33 days. Flowers and siliques are already visible in Ler when bolting occurs in rug1. Bar = 1 cm. (b, c) Flowering time, determined as (b) the total leaf number (rosette and cauline leaves from the main inflorescence) and (c) the number of days for bolting. Both Ler and rug1 were grown under continuous light and vernalized for 4 weeks (Vernalization +) or just stratified (Vernalization −) before being transferred to our standard growth conditions. Values are means and standard errors for 20 plants. Asterisks indicate rug1 values significantly different from those of Ler (Students t-test, P<0.01). (d) qRT-PCR analysis of the expression of the FLC, FT and SOC1 genes in the rug1 mutant.
Bars indicate relative levels of expression, determined as $2^{-\Delta \Delta Ct}$, for each of the studied genes after normalization with those of the housekeeping gene G3PDH and also normalized to the values obtained for Lr, to which a value of 1 was given. All quantifications were made in triplicate on RNA samples. Plant material for qRT-PCR was collected 21 days (PPT).

Figure S2 Complementation of the mutant phenotype of rug1 and effects of RUG1 overexpression in a wild-type genetic background. (a–c) Rosettes of (a) the rug1 mutant, (b, c) transgenic plants carrying the S35SRUG1 transgene in a rug1 background, (b) one of which is phenotypically wild type while (c) the other does not show any of the mutant phenotypic traits that characterize rug1 and develops many vegetative leaves, apparently as a consequence of shoot apical meristem duplication; (d, e) The phenotype shown in (c) was also caused by expression of the S35SRUG1 transgene in a Ler background (RUG1). (f) Some of these S35SRUG1 RUG1 transgenic plants exhibited some necrotic spots. Pictures were taken (a, b) 21 days, (c) 29 days and (d–f) 26 days. Bars = 1 mm. (PPT)

Figure S3 Effect of different light conditions on the phenotype of the rug1 mutant. Rosettes of (a–c) Ler and (d–f) rug1 grown under (a, d) continuous light, (b, e) long day conditions (16-h light/8-h dark) and (c, f) 15 days in long day conditions followed by 8 days of continuous light. Pictures were taken at 23 days. Bars = 1 mm. (PPT)

Figure S4 Physiological analyses of the rug1 mutant. (a) Moderate light sensitivity of rug1 as seen by growing Ler (upper panels) and rug1 (bottom panels) under low (35 μmol m$^{-2}$ s$^{-1}$) or high (115 μmol m$^{-2}$ s$^{-1}$) levels of visible light. Arrows indicate enhanced necrotic lesions in rug1 after exposure to high light intensities. (b) Skotomorphogenic growth is not altered in rug1. The histogram shows means (n≥15) and standard deviations of hypocotyl length in rug1, lin2 and their respective wild types, Ler and Col-0, grown in the dark for 10 days. Seedlings of the aba1-1 and aba1-101 mutants (in a Ler and Col-0 genetic background, respectively) were included as controls since they are known to be partially defective in the skotomorphogenic response. The lin2 mutant is deficient in the coproporphyrinogen III oxidase enzyme, which acts downstream of PBGD in the tetrapyrrole pathway (Figure 1). The rug1 and aba1-1 mutants are in the Ler genetic background. lin2 and aba1-101 are in the Col-0 genetic background. (c) Root growth inhibition by IAA. Each point represents mean data (n≥15) of the reduction in root length displayed by plants grown on media supplemented with the IAA concentrations shown, compared with those grown on non-supplemented media. Error bars indicate standard deviations. Asterisks indicate rug1 values significantly different from those of the wild type (Students t-test, P<0.01). (d) Effects of sucrose on rug1 growth. Ler (upper-left panel) and rug1 (upper-right panel) plants grown in the absence of sucrose are shown. The bar graph represents the percentage of plants with arrested development in the absence of sucrose. Data are means of two different replicates of 50–100 seeds each, scored at 21 days. An arrested rug1 seedling is marked by a red circle. Bars = (b) 1 mm and (d) 5 mm. (PPT)

Figure S5 Catalase activity in the rug1 mutant. Box plots showing catalase activity, expressed in enzyme units (U) per mg of protein. Samples were obtained from 21-day-old rosettes of the rug1 mutant and its wild type Ler, grown under continuous light or long day conditions (16-h light/8-h dark). Each box plot was obtained from the values of 3–6 measurements. (PPT)

Figure S6 GOriLLa analysis output of rug1 misregulated genes. GO term enrichment for (a) down-regulated and (b) upregulated genes using the Biological Process ontology is represented. Two unranked lists were used for enrichment calculations, consisting in genes represented in the microarray and recognized by the GOriLLa database (18,726 in this study), and genes found down-regulated (103) or up-regulated (155) in the rug1 mutant. Enrichment was calculated as (b/n)/B/N). N: total number of genes in the reference set (microarray) associated with any GO term (16,222); B: number of genes in target set (64 and 73 down- and up-regulated genes, respectively, in the rug1 microarray) associated with a GO Process; n: total number of genes in the microarray associated with a specific GO term, and b: number of (a) down- or (b) up-regulated genes in the rug1 microarray associated with a specific GO term. Colors reflect the degree of GO term enrichment as indicated in the legend. A P-value of 10$^{-5}$ was used as threshold. (PPT)

Table S1 Functional classification of misregulated genes in rug1.

(PDF)

Table S2 Primers used in this work.

(PDF)

Acknowledgments

We wish to thank H. Candela and P. Robles for their useful comments on the manuscript, the NASC for provision of seeds, R. Solano for microarray analyses, and J.M. Serrano, T. Trujillo, V. García-Sempere and L. Serna for their excellent technical assistance.

Author Contributions

Conceived and designed the experiments: VQ MRP JLM. Performed the experiments: VQ RSM Rgb AH. Analyzed the data: VQ MRP JLM. Contributed reagents/materials/analysis tools: MRP JLM. Wrote the paper: VQ JLM MRP.

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