The Greening after Extended Darkness1 Is an N-End Rule Pathway Mutant with High Tolerance to Submergence and Starvation1[OPEN]

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Plants respond to reductions in internal oxygen concentrations with adaptive mechanisms (for example, modifications of metabolism to cope with reduced supply of ATP). These responses are, at the transcriptional level, mediated by the group VII Ethylene Response Factor transcription factors, which have stability that is regulated by the N-end rule pathway of protein degradation. N-end rule pathway mutants are characterized by a constitutive expression of hypoxia response genes and abscisic acid hypersensitivity. Here, we identify a novel proteolysis (prt6) mutant allele, named greening after extended darkness1 (ged1), which was previously discovered in a screen for genomes uncoupled-like mutants and shows the ability to withstand long periods of darkness at the seedling stage. Interestingly, this ethyl methanesulfonate-derived mutant shows unusual chromosomal rearrangement instead of a point mutation. Furthermore, the sensitivity of N-end rule pathway mutants ged1 and prt6-1 to submergence was studied in more detail to understand previously contradicting experiments on this topic. Finally, it was shown that mutants for the N-end rule pathway are generally more tolerant to starvation conditions, such as prolonged darkness or submergence, which was partially associated with carbohydrate conservation.

During their life cycle, plant cells encounter periods of oxygen deficiency, generally termed as hypoxia stress. This stress depends on the ratio between oxygen consumption and availability. In plants, oxygen diffusion can be constrained by anatomical and morphological features (i.e. densely packed cells [e.g. meristems] and a low surface-to-volume ratio [e.g. fruits and seeds]) or external conditions that hamper gas diffusion, such as complete submergence of plants or waterlogging of soils (root flooding). Because of the requirement of oxygen for biosynthetic processes and more importantly, mitochondrial respiration, a shortage in oxygen leads to an energy deficit in hypoxic cells. To cope with this stress, plants reprogram their metabolism involving down-regulation of energy-demanding processes (e.g. protein biosynthesis) and up-regulation of genes important for glycolysis, fermentation, and protection against reactive oxygen species (Bailey-Serres and Voesenek, 2008; Branco-Price et al., 2008; Mustroph et al., 2009; Voesenek and Bailey-Serres, 2013).

The transcriptional regulation of flooding-adaptive genes operates through a specific signal transduction pathway triggered by a decline in the cellular oxygen concentration. This oxygen-sensing mechanism is mediated by the oxygen- and nitric oxide-dependent degradation of subgroup VII Ethylene Response Factor (VII-ERF) transcription factors through the N-end rule pathway of proteolysis (Gibbs et al., 2011, 2014; Licausi et al., 2011). Stabilization of VII-ERFs (among them, RELATED TO AP2 2 [RAP2.2] and RAP2.12) during hypoxia activates the transcription of downstream hypoxia-responsive genes, including those involved in fermentation and sugar consumption (Gibbs et al., 2011; Licausi et al., 2011). During reoxygenation or normoxia, these transcription factors are modified in the presence of oxygen at the N-terminal Cys, most likely through the action of Plant Cys Oxidase (PCO1/PCO2; Weits et al., 2014), making them accessible to protein degradation through the N-end rule pathway through arginylation:protein arginyltransferase (ATE1/ATE2) and the E3 ubiquitin ligase PROTEOLYSIS6 (PRT6). Mutants for either ate1/ate2 or prt6 are disturbed in degradation of VII-ERFs and consequently, accumulate these proteins even under oxygen-rich conditions, therefore exhibiting a constitutive hypoxia response (Gibbs et al., 2011, 2014; Licausi et al., 2011).

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RESULTS

ged1 Displays a Hypoxic Expression Pattern under Normoxia and Possesses a Nonfunctional PRT6 Gene

The ged1 mutant has been initially characterized as a mutant that exhibits the ability to green after extended periods of darkness and additionally, shows sensitivity of germination to ABA (Choy et al., 2008). The ged1 mutant is characterized by a down-regulation of many ABA-responsive genes when grown for 5 d in darkness followed by 2 d in light (Choy et al., 2008) and an up-regulation of many hypoxia-responsive genes (Fig. 1A). Of 408 significantly induced genes (signal log ratio > 1, adjusted \( P < 0.05 \)), 40% were hypoxia responsive (Supplemental Table S1; Branco-Price et al., 2008; Mustroph et al., 2009; Gibbs et al., 2011; Licausi et al., 2011), and 36 of 49 hypoxia core response genes (Mustroph et al., 2009) were among these up-regulated genes. For example, many genes for hypoxic metabolism (alcohol dehydrogenase, pyruvate decarboxylase [PDCl], Suc synthase1 [SUS1], and SUS4) as well as genes coding for HYPOXIA-RESPONSIVE UNKNOWN PROTEINS (Mustroph et al., 2010; Lee et al., 2011) were highly expressed in ged1 under normoxic conditions. However, not all well-known hypoxic genes were among the significantly highly expressed genes in ged1, including the hypoxia-responsive phosphofructokinase3 (PFK3) and PFK6, Ala aminotransferase1, RESPIRATORY BURST OXIDASE HOMOLOG D, and SIMILAR TO RCD ONE5. In addition, the down-regulated genes in ged1 were often ABA responsive, which has already been observed previously (Choy et al., 2008); 161 of 540 significantly down-regulated genes were up-regulated in ABA-treated seeds or seedlings from published microarray experiments (Fig. 1C; Supplemental Table S1; Goda et al., 2008; Kim et al., 2011).

In a subsequent analysis, the constitutive expression of hypoxic genes was confirmed on 7-d-old ged1 and WS wild-type seedlings by PCR analyses (Fig. 1D). To identify the ged1 mutation, we performed map-based cloning based on the ABA-hypersensitive arrest of seedling establishment. F2 seeds of a cross between ged1 (background WS) and Col-0 were grown on MS medium containing 0.1 \( \mu \)M ABA. About 16.4% of all F2 seedlings showed the mutant phenotype and did not develop green cotyledons (Fig. 2A). They were rescued by transfer to ABA-free medium and subsequently used for genotyping. ABA hypersensitivity of selected lines was confirmed by analyzing the F3 generation.

The first rough mapping process with 26 loci across all five chromosomes revealed the existence of two cosegregating loci: one on chromosome 3 and one on chromosome 5. Fine mapping of the two regions with additional markers specified the regions on chromosome 3 between 2.6 and 3.3 million bp and on chromosome 5 between 1.0 and 2.7 million bp. All plants that showed the mutant phenotype and were evidently not segregating in the F3 generation were homozygous for the mutant genotype (WS) on top of chromosome 5. However,
no recombination event could be detected in the first 2.7 million bp, despite a mapping population of about 1,000 plants. None of the plants with the mutant phenotype were homozygous for the wild-type genotype Col-0 in the second cosegregating region on chromosome 3, but about 20% were heterozygous.

Genotyping of individuals from different F3 generations that were derived from those plants that were heterozygous on chromosome 3 in the F2 generation and showed the mutant phenotype without segregation in the F3 generation revealed an abnormal segregation pattern in the mapped region on chromosome 3, with mostly homozygous WS genotype and few heterozygous and no homozygous Col-0 genotype plants. Therefore, the pattern of genotype distribution between the mapped regions on chromosomes 3 and 5 was analyzed in 91 F2 plants without previous phenotype selection. One representative marker was used for each region (3367348_bp and NGA249 for chromosomes 3 and 5, respectively). No plants with the genotype combination homozygous Col-0 and homozygous WS on both chromosomes were found, and all possible heterozygous/ homozygous combinations were less frequent, which was not to be expected by random distribution (Fig. 2B).

In contrast, combinations of the same genotype at both chromosome regions were more frequent. For example, the combination of homozygous WS at both loci was detected in 16.5% of all F3 plants.

After microarray data of *prt6* and *ate1/ate2* T-DNA insertion null alleles were published (Gibbs et al., 2011; Licausi et al., 2011), we became aware of similarities with these mutants and *ged1* regarding the regulation of hypoxia-responsive genes and ABA sensitivity (Fig. 1B; Supplemental Table S1). Additionally, all three genes were close to the mapped regions on chromosome 3 (*ATE2*) or chromosome 5 (*ATE1* and *PRT6*). Therefore, crosses of these genotypes with *ged1* were produced, and the F1 generation was examined for germination on ABA-containing medium. Seedlings of a cross between *ged1* and *prt6-1* but not between *ged1* and *ate1/ate2* showed ABA hypersensitivity during germination (Fig. 2C). Hence, we concluded that *ged1* bears a nonfunctional PRT6 gene.

To identify the position of the mutation and confirm our hypothesis, we PCR amplified and sequenced a nearly 10,000-bp-long genomic region around the PRT6 gene in about 2,000-bp sections. However, one primer combination spanning a certain region of the
The PRT6 gene did not yield a PCR product in genomic DNA of ged1 but worked well in the WS wild type. To identify the cause of this PCR problem, the region was specified with more primer pairs, and finally, thermal asymmetric interlaced (TAIL) PCRs starting from both sites of this region were performed. This revealed that the first part of PRT6 directly merges into the second part of the gene MYB DOMAIN PROTEIN 3R-3 (MYB3R3; At3g09370), which is located within the cosegregating region on chromosome 3. In the other direction, the second part of PRT6 directly merges into the first part of MYB3R3 (Fig. 2D; Supplemental Figs. S1 and S2). These results suggest that one part of PRT6 moved to chromosome 3 and one part of MYB3R3 moved to chromosome 5.

Two approaches were followed to estimate the extent of chromosome exchange. (1) Regions around PRT6 and MYB3R3 were sequenced, and primers creating overlapping DNA sequences were used to determine if it is possible to yield PCR products of roughly 4,000 bp upstream and downstream of the genes. (2) Because the recombination rate on top of chromosome 5 was very low, F2 plants with the mutant phenotype (homozygous WS on top of chromosome 5) that showed recombination events (heterozygous/WS) near to the cosegregating region on chromosome 3 were analyzed. All plants that were homozygous WS within at least 2.8 and 3.3 million bp on chromosome 3 but heterozygous outside of this region showed normal seed yield and a normal segregation pattern (i.e. in the F3 generation, homozygous Col-0 plants were detected with mapping markers in the heterozygous parts of the respective F2 plants). However, when plants were heterozygous within 2.8 and 3.3 million bp, they had small siliques with low seed yield, and homozygous Col-0 plants could not be detected in the F3 generation. We, therefore, assume that the DNA exchange on chromosome 3 should be restricted to these 0.5 million bp.

The combination of both approaches did not, however, result in the detection of a second point of chromosome exchange. Potentially, a large chromosome part could have been transferred. Furthermore, this chromosome transfer explains the unusual non-Mendelian segregation of the F2 generation (Fig. 2B), making several combinations of loci between Col-0 and ged1 lethal because of missing chromosome fragments.

In addition to PRT6, the other two N-end rule pathway genes ATE1 and ATE2 were also sequenced in ged1 to exclude additional mutations. The sequencing resulted in WS wild-type-like ATE1 and ATE2 genes in the mutant ged1. Surprisingly, a single-nucleotide insertion in both WS ecotype and ged1 compared with the Col-0 reference
sequence was observed for ATE2, leading to a premature stop codon and a truncated, presumably nonfunctional ATE2 protein (Supplemental Figs. S3–S5). It is, therefore, concluded that WS only contains one functional ATE gene. This is further supported by the analysis of the F2 generation of our WS × ate1/ate2 cross. Here, 25 plants showed ABA-sensitive germination, and 65 plants did not. This 1:3 segregation rate hints at only one functional ATE gene.

**Survival of prt6 Mutants under Submergence**

Previous work describing the submergence/hypoxia tolerance of prt6 revealed partially contrasting results (Gibbs et al., 2011; Licausi et al., 2011). Because we discovered that ged1 is another allele of a prt6 mutant, we determined the submergence tolerance of ged1. The submergence assay was done as described by Vashisht et al. (2011) under illumination or darkness. This assay showed that ged1 is highly tolerant to submergence in darkness or submergence under short-day light conditions compared with its wild type (median lethal time \( LT_{50} \) = 14.0 versus 11.8 d and 26.3 versus 18.6 d under darkness and light, respectively; Fig. 3; Table I). In contrast, LT\(_{50}\) values for air dark conditions of ged1 and the wild type were only marginally different (LT\(_{50}\) = 12.4 versus 11.7 d). Remarkably, ged1 mutants survived the compound stress of submergence (dark) better than darkness alone. Although 50% of the ged1 plants survived 14 d of darkness during submergence, only 10% survived 14 d of darkness in air. In both cases, no wild-type plants survived.

Because ged1 is a prt6 knockout (KO) mutant, our results for survival of submergence in darkness are contradictory to the published work by Licausi et al. (2011), who examined a prt6 T-DNA insertion line (named here as prt6-6; Supplemental Table S2) in a WS background and found it to be more intolerant than the corresponding wild type to 3.5 d of submergence in darkness. We repeated our experiments with prt6-1 (Garzón et al., 2007; Graciet et al., 2009; Supplemental Table S2) and its wild-type Col-0. In these experiments, prt6-1 is clearly more tolerant than Col-0 to both darkness (in air) and submergence (under dark and short-day light conditions; LT\(_{50}\) = 10.0 versus 6.1 d and 22.2 versus 16.2 d under submerged darkness and submerged light, respectively; Fig. 3; Table I). In contrast to the results by Licausi et al. (2011), 100% of the prt6-1 and ged1 plants survived 8 d of submergence in darkness in our experimental setup.

A comparison of both ecotypes revealed that the WS wild type is more tolerant than Col-0 under all treatment conditions. Interestingly, prt6-1 had a higher LT\(_{50}\) and therefore, a better survival when treated with darkness in air compared with darkness under submergence, whereas the opposite was observed in ged1, indicating important ecotype differences as previously observed (Vashisht et al., 2011).

To understand the contrasting results between both experiments, we looked more deeply into the growth and treatment conditions (Licausi et al., 2011; Vashisht et al., 2011). One major difference between both survival assays is the relative humidity levels of the recovery period. Although in our conditions, the relative humidity during the recovery phase was maintained at 70% (Vashisht et al., 2011), it was much lower (presumably lower than 30%) in the other experimental setup (F. Licausi, personal communication). This difference could have caused the lower overall survivability of plants in the study by Licausi et al. (2011) as well as the difference in sensitivity in the prt6-6 T-DNA insertion line compared with the wild type. To test this hypothesis, we performed an experiment, in which prt6 mutants were allowed to recover after submergence at either a low humidity (about 20%) or a high humidity (about 100%). Under the low-humidity recovery conditions, only a few plants survived 5 d of submergence, but all plants survived and looked much healthier when recovery was performed at high humidity (Supplemental Fig. S6). Furthermore, more wild-type plants than prt6 mutant plants survived when recovered at low humidity (such as in Licausi et al., 2011), whereas more prt6 mutant plants than wild types survived during high-humidity recovery.

![Figure 3](https://plantphysiol.org)

**Figure 3.** ged1 and prt6 are more tolerant to submergence than their respective wild types. Soil-grown 5-week-old plants were submerged for the indicated time points in complete darkness (SUB-DARK) or under a day-night regime (SUB-LIGHT) and assessed for survival exactly as described in Vashisht et al. (2011). Additionally, plants were treated with darkness in air for comparison (AIR-DARK). LT\(_{50}\) values and survival estimates were calculated according to the Weibull regression model as described in Vashisht et al. (2011). LT\(_{50}\) values ± se are presented in Table I.
with the respective differences in tolerance, and (2) the wild type compared to the two ecotypes Col-0 and WS that showed huge differences in carbohydrate conservation and/or differences in the tolerance to water deficiency stress. More vital-looking leaves directly after desubmergence as well as better recovery under high humidity indicate better shoot survival of prt6 mutants during submergence compared with the respective wild type (Supplemental Fig. S6).

### Prolonged Submergence Survival of prt6 Mutants Is Associated with Carbohydrate Conservation

Gene expression analyses of ged1 and other prt6 mutants show a constitutive up-regulation of genes involved in fermentation and sugar breakdown (Choy et al., 2008; Gibbs et al., 2011). However, a higher percentage of ged1 survived during submergence in the dark than in darkness only (Fig. 3). This contradicted our intuitive assumption that carbohydrate resources would be wasted during submergence because of fermentative metabolism, which has been previously suggested (Licausi et al., 2011). Therefore, we addressed the question of how far the difference in tolerance might be reflected by a difference in primary metabolism in: (1) the two ecotypes Col-0 and WS that showed huge differences in tolerance, and (2) the wild type compared with the respective prt6 knockout mutants.

Hence, 5-week-old soil-grown plants (8-h-d/16h-night cycle) were submerged in darkness for 8 and 24 h at the end of the light period, so that the plants went into the stress treatment with a maximal amount of carbohydrate reserves. The sugar content, including Glc, Suc, and starch, was similar at the start of the stress treatments in all tested genotypes (Fig. 4A; Supplemental Table S3B). Fru levels were significantly elevated in the ged1 and prt6-1 compared with their respective wild types at the start of the stress treatment but only contribute 0.6% to 1.8% to the total sugar content.

After 8 h of submergence in darkness, Glc and starch levels significantly decreased in all genotypes compared with the starting level, whereas the changes in the other two sugars were not significant (Fig. 4B; Supplemental Table S3A). No significant differences between the genotypes were observed; 8 h of darkness in air also significantly decreased all sugars analyzed, with the exception of Suc and Fru in Col-0, and again, no clear differences between genotypes were visible. Submergence versus air treatment did not show a difference in sugar levels within each genotype, except for a comparatively small but significant elevation in Fru levels after submergence in both prt6 mutants (Supplemental Table S3B). ATP and ADP levels also did not differ significantly among treatments and genotypes (Supplemental Fig. S7).

Similarly, 24 h of submergence in darkness led to lower sugar levels in all genotypes, except for Fru (Supplemental Table S3A). However, higher amounts of starch remained in both prt6 mutants compared with their respective wild types and air treatment (Fig. 4D; Supplemental Table S3B). The difference in starch content between 24 h of submergence and air treatment was about 6-fold in prt6-1 and 10-fold in ged1. The prt6-1 T-DNA insertion line additionally showed significantly higher Suc and Fru contents compared with the Col-0 wild type. In this regard, no significant difference was measured between WS and ged1. Interestingly, no difference between all genotypes was observable after 24 h of darkness in air. Adenylates again showed no significant differences, such as after 8 h of submergence (Supplemental Fig. S7).

In addition to submergence, we also treated the plants with a pure nitrogen atmosphere in darkness (anoxia) for 8 h. This treatment resulted in highly decreased starch degradation compared with air dark treatment without any noticeable difference between genotypes and a high accumulation of Glc, whereas Suc values approximated the detection limit. An ecotype-specific difference was observable for the Glc levels after anoxia treatment, with higher levels in Col-0 than WS genotypes (Fig. 4C; Supplemental Table S3B).

#### prt6 Mutants Are Tolerant to Starvation Conditions

In the first description of the ged1 mutant, its ability to show increased greening after extended darkness...
was discovered (Choy et al., 2008). Here, we wanted to confirm this finding and extend it to an independent *prt6* T-DNA insertion line. In contrast to WS genotypes, the Col-0 genotypes were not able to green after 10 d of dark germination. For these genotypes, 8 d of dark germination were applied. For both *prt6* alleles, however, we could clearly show the better ability to green after extended darkness compared with the respective wild type (Fig. 5). During our analyses, it was also confirmed that Suc supplementation of the medium interrupted this phenotypic difference (Supplemental Fig. S8).

In addition to germination under darkness, we tested another starvation condition. It had been previously shown that *ate* mutants exhibit delayed leaf senescence (Yoshida et al., 2002; Holman et al., 2009). When 7-d-old light-grown seedlings were transferred to darkness for 9 to 12 d, the wild-type plants did not survive this treatment, whereas *prt6-1* and *ged1* were still able to recover from this treatment (Fig. 5).

Leaf Respiration Rates Are Not Different in *prt6* Mutants

The observations above (Figs. 4D and 5; Supplemental Fig. S6) led us to assume the presence of lower respiration rates in *ged1* and *prt6-1*. However, neither rosette leaves after 24 h of submergence nor seedlings after normal growth or short-term starvation (additional 6 h of darkness treatment shortly after the night phase) showed a difference in oxygen consumption between *prt6* mutants and the respective wild types under normoxic conditions (Fig. 6). In planta measurements of respiration rates during the whole stress period would be required to answer this question.

DISCUSSION

The *ged1* Mutant Is a Novel *prt6* Allele

A phenotype of the mutant *ged1* (Choy et al., 2008) was an increased expression of hypoxic genes under normal oxygen conditions (Fig. 1), showing the importance of this mutation in hypoxia signaling. The mapping process revealed a mutation in the previously characterized gene *PRT6* (*At5g02310*), a component of the N-end rule pathway of protein degradation that is important for hypoxic signal transduction (Gibbs et al., 2011; Licausi et al., 2011). Interestingly, the mutation was not a simple nucleotide exchange...
caused by the EMS treatment but an exchange of larger chromosomal sequences between chromosomes 3 and 5. This chromosome exchange occurred between two syntenic regions on these chromosomes containing 108 anchors (chromosome 3: At3g09300–At3g12320 and chromosome 5: At5g02100–At5g06980), including ATE1 (At5g05700), ATE2 (At3g11240), and the transcription factors MYB3R3 (At3g09370) and MYB3R5 (At5g02320; Supplemental Fig. S9; Lee et al., 2013; http://chibba.agtec.uga.edu). MYB3R3 is the gene where the chromosome exchange in ged1 occurred on chromosome 3, whereas its syntenic gene MYB3R5 is a direct neighbor of PRT6. One can only speculate that the EMS reagent caused a strand break and chromosome rearrangement because of an alignment of closely related but not homologous regions during cell division.

Although a chromosome exchange because of EMS treatment is not common, this effect has been reported several times for animals and plants (Moutschen et al., 1964; Natarajan and Upadhya, 1964; Cattanach et al., 1968; Sega, 1984). Such mutations have not been discovered often in Arabidopsis because of the experimental difficulties in detecting them.

The affected gene PRT6 was confirmed by the analysis of a cross between prt6-1 and ged1, showing the same ABA-sensitive germination phenotype in the F1 generation as in both parental lines (Fig. 2). Furthermore, the various phenotypes of prt6 T-DNA insertion null alleles and ged1 are similar (for example, the ABA-sensitive germination [Choy et al., 2008; Holman et al., 2009], the ability to green after extended darkness [Choy et al., 2008; Fig. 5], and the high expression of hypoxic core genes [Choy et al., 2008; Gibbs et al., 2011]).

Analysis of the sequences of the other N-end rule pathway genes, ATE1 and ATE2, revealed that the WS ecotype only has one functional ATE gene, ATE1, which we showed by sequencing and analysis of the F2 generation of the WS × atel1/ate2 cross. Our finding is further supported by a previously identified mutant, delayed leaf senescence1, which shows delayed leaf senescence and has only a mutated ATE1 gene in the WS background (Yoshida et al., 2002). This delayed leaf senescence is similar to the phenotype of prt6 mutants that we show here (Fig. 5), and the mutant confirms that ATE2 had to be nonfunctional in this ecotype. In Col-0, both genes have to be knocked out to show a similar severe phenotype (Graciet et al., 2009; Holman et al., 2009).

Other than PRT6, ged1 also contains a nonfunctional MYB3R3 gene, which is, to our knowledge, uncharacterized until now. However, we do not expect a phenotype from the mutation of MYB3R3, because Arabidopsis has the closely related ortholog MYB3R5, which is still intact in ged1 and shares about 51% identity at the amino acid level with MYB3R3. It is most likely that the defect in MYB3R3 can be replaced by MYB3R5.

**Figure 5.** Survival of seedlings under dark germination and darkness treatment. Seeds of Arabidopsis lines (Col-0, prt6-1, WS, and ged1) were either germinated in darkness for 10 (A) or 8 d (B) and then allowed to recover for another 7 d, or seeds were germinated under long-day conditions for 7 d and then subjected to a darkness treatment for 12 d (C and D) followed by a 7-d recovery. E and F, Quantification of survival experiments. Number of surviving seedlings after treatment and recovery was related to the total number of seedlings. Values are means ± se of three to six independent experiments. Different letters show significant differences as determined by Tukey’s honestly significant difference mean separation test (P < 0.05).
Hypoxic Survival of *prt6* Mutants

After the identification of *ged1* as another *prt6* allele, we investigated its sensitivity toward oxygen deficiency stress because of contradicting reports on the tolerance of *prt6* T-DNA insertion null alleles to hypoxia and submergence (Gibbs et al., 2011; Licausi et al., 2011). Hypoxia survival experiments with Arabidopsis are usually carried out under rather controlled environmental conditions (for example, compared with field experiments). However, a wide range of different hypoxia survival experiments exist in the literature, and especially, submergence survival experiments vary greatly in the length of survival without any obvious reasons (for example, Banti et al., 2010; Licausi et al., 2011; Vashisht et al., 2011). In case of submergence in darkness, for example, different publications report great differences in the length of survival (i.e. Col-0 has an LT50 of about 8 d with 100% survival after 5 d of submergence [Vashisht et al., 2011], whereas only about 70% of plants from the same genotype survived 3.5 d of submergence in another experiment [Licausi et al., 2011]). Furthermore, factors like developmental stage, sugar supplementation, oxygen concentrations, hypoxic pretreatment, illumination, and genotypic background have been shown to greatly influence the length of survival and have an impact on the measured tolerance or intolerance of transgenic plants (Ellis et al., 1999; Hunt et al., 2002; Ismond et al., 2003; Loreti et al., 2005; Banti et al., 2008; Vashisht et al., 2011).

In our experiments, we report that two *prt6* mutant alleles show better submergence survival compared with their respective wild types under two different illumination conditions (Fig. 3; Table 1). These results confirm the findings by Gibbs et al. (2011) showing higher hypoxia tolerance of the *prt6-1* T-DNA insertion line. With respect to the contradicting results of other studies (Licausi et al., 2011; Weits et al., 2014), we propose that the air humidity during the recovery period is a crucial factor for the length of survival of submergence in darkness, which was shown here for treatment of the different genotypes for 5 and 7 d of submergence in darkness (Supplemental Fig. S6). The humidity during recovery not only influenced overall survivability of plants but also, caused opposite behavior of *prt6* mutants versus wild-type plants. These observations hint at different reasons for plant death under different treatment conditions. Root damage caused by submergence itself impairs water uptake and leads to dehydration and wilting of leaves during low-humidity recovery, which seems to be the ultimate cause of death under this condition. Differences in tolerance to low-humidity recovery could possibly be influenced by the length of root survival and/or differences in the adaptation to water deficiency stress (e.g. stomatal closure; Gibbs et al., 2014). Tolerance to submergence followed by high-humidity recovery, however, seems to be rather independent of these tolerance factors. Together with our analysis of carbohydrate levels in shoots of *prt6* mutants after submergence in darkness (as discussed below; Fig. 4), one could argue that they survive submergence followed by high-humidity recovery better because of a more efficient carbohydrate usage in leaves and delayed damages related to carbohydrate starvation. However, at the same time, this observation might suggest a reduced carbohydrate supply to the roots, leading to faster death of this organ and therefore, faster death during low-humidity recovery. More experiments are required to fully understand these survival differences.

A very important aspect of the submergence in darkness experiments by Vashisht et al. (2011) is that the LT50 values are compared with survival of darkness in air by the calculation of a hazard ratio, which is a measure of the difference in the respective length of survival. Interestingly, this shows that the majority of
86 examined ecotypes survived darkness in air longer than under submergence (positive hazard ratio) but that some ecotypes are able to survive darkness during submergence as long as or even longer than in air under similar growth and recovery conditions (negative hazard ratio). We show here that the WS wild type of ged1 is one of those ecotypes that survives darkness in air as long as during submergence (Table I), similar to the WS-0 and WS-2 ecotypes (Vashisht et al., 2011) that are genotypically only slightly different from the wild type of ged1. Amazingly, ged1 exceeds survival of darkness during submergence compared with its wild type by more than 2 d and is the most tolerant genotype that has ever been measured in these survival experiments. However, ged1 survives prolonged darkness only about as long as the wild type in air, resulting in a negative hazard ratio. In contrast, the Col-0 wild type and prt6-1 have positive hazard ratios, with a clearly higher LT50 value of prt6-1 than Col-0 both in air and during submergence (Table I). The reason for the similar or better survival of dark submergence than darkness alone for ged1, WS, and the ecotypes that share a negative hazard ratio is, however, still unknown. The sugar content in leaves does not differ between the ecotypes after 24 h of darkness (Fig. 4D) as well as the aerobic dark respiration rates (Fig. 6), but other factors could be involved, such as the efficient usage of other energy sources (e.g. lipids).

Different causes of death might have occurred in the experiments presented here (Fig. 3), among them death from sugar starvation, death from root damages, death from reactive oxygen species during desubmergence, and other factors. This hypothesis is being underlined by our starch and sugar measurements after 24 h of submergence in darkness, which reveal a higher total sugar content in prt6-1 compared with the WS ecotype (Fig. 4D), whereas WS survived submergence better than prt6-1 (Fig. 3). This indicates a different cause of death in prt6-1 than leaf carbohydrate starvation. In contrast, the higher total sugar content of ged1 compared with WS gives an explanation for the negative hazard ratio and its superior survival rate. This indicates that not only do different hypoxia survival experiments measure different causes of death, but also, in the very same survival experiment, different causes of death are measured in different genotypic backgrounds. In this regard, it is interesting that a positive effect of hypoxia pretreatment has been shown for the C24 ecotype in previous publications (Ellis et al., 1999; Hunt et al., 2002; Ismond et al., 2003). The same ecotype has a high negative hazard ratio in the publication by Vashisht et al. (2011) and also, shows the highest LT50 of survival of submergence in darkness among 86 tested accessions.

The importance of carbohydrates for survival of oxygen deficiency stress has been also shown in experiments with anoxia or severe hypoxia, such as those carried out, for example, by Gibbs et al. (2011). It has been shown that survival of Col-0 seedlings can be prolonged from less than 1 to 3 d simply by a triplication of Suc supplementation (90 mM compared with 30 mM; Banti et al., 2008). Similar results were published for Col-0 glabra1 (Loreti et al., 2005), indicating that carbohydrate starvation greatly contributes to the cause of death in these survival experiments. In this context, our sugar measurements after 8 h of nitrogen gas treatment in darkness reveal a high accumulation of Glc together with reduced starch consumption compared with air treatment (Fig. 4C). This suggests that the first energy-consuming step for the degradation of Glc through glycolysis by hexokinases is hampered because of a bioenergetic collapse of the cells. In contrast, Suc levels that were at a concentration of about 2 μmol g−1 of fresh weight at the start of the treatment approximated the detection limit after 8 h of stress treatment, confirming observations that Suc but not Glc supplementation results in prolonged anoxia survival of Col-0 seedlings and is associated with a higher fermentation rate (Banti et al., 2008). Similar observations have been reported for tomato (Solanum lycopersicum) roots (Germain et al., 1997). Most likely, energy-efficient cleavage of Suc by Suc synthases is favored under anoxic conditions rather than starch and Glc consumption (Bailey-Serres and Voesenek, 2008).

The Submergence Tolerance of prt6 Mutants Is Associated with Sugar Conservation

When oxidative phosphorylation is hampered, a reduction of energy-consuming processes is inevitable. Several evidences for a down-regulation of energy consumption in response to hypoxia exist. In both plants and animals, this happens by a down-regulation of translation (Branco-Price et al., 2008; Mustroph et al., 2009; Wheaton and Chandel, 2011). Furthermore, down-regulation of other biosynthetic processes (for example, storage metabolism; Geigenberger, 2003) and growth processes (for example, root growth of Arabidopsis; van Dongen et al., 2009) have been observed.

In our experiments, treatment with darkness expectedly reduced sugar and starch levels in Arabidopsis leaves (Fig. 4). However, higher total sugar levels were detected after 24 h of submergence in darkness compared with air in darkness in rosette leaves of all genotypes, indicating that our submergence treatment triggered a reduction in carbohydrate consumption. No differences in total sugar levels were observable after 8 h, which could be because of the remaining air inclusions inside the leaves or slowly decreasing oxygen partial pressures in the water column or soil that become anoxic only after 24 h (Vashisht et al., 2011). Interestingly, the reduction in carbohydrate consumption after 24 h was significantly stronger in prt6 mutants than the respective wild type (Fig. 4D). Remarkably, the difference in starch content between 24-h submergence and air treatment was about 6-fold in prt6-1 and 10-fold in ged1, whereas no significant difference in starch content between both treatments could be observed in the wild types (Supplemental Table S3). In contrast, no differences in sugar or starch levels were
observable between the genotypes after darkness in air treatment.

The lower sugar consumption rate in prt6 mutants under submergence compared with wild-type plants is not readily expected, because the potential of sugar consumption is expected to be much higher because of a higher expression of genes involved in fermentative metabolism in the mutants, at least at the start of the treatment (Fig. 1; Choy et al., 2008; Gibbs et al., 2011). Interestingly, hypoxia-inducible SUS genes SUS1/SUS4 but not PFK genes PFK3/PFK6 are among the highly expressed genes in prt6 mutants (Supplemental Table S1; Choy et al., 2008; Gibbs et al., 2011), which could influence the glycolytic rate under submergence. A generally high fermentation capacity does not always result in an increased rate of fermentation and a higher submergence survival. Although PDC overexpression lines of Arabidopsis showed enhanced hypoxia tolerance (Shiao et al., 2002; Ismond et al., 2003), experiments with other plant species showed the opposite effect (Tadege et al., 1998; Rahman et al., 2001). Furthermore, rice (Oryza sativa) plants expressing the transcription factor Submergence 1A exhibited enhanced expression of fermentative genes but lower fermentation rates and higher submergence survival than other genotypes (Fukao et al., 2006). It is, therefore, concluded that high expression of fermentative genes in prt6 mutants does not necessarily lead to higher sugar consumption and fermentation rates under submergence. Rather, the carbohydrate status hints at a lower fermentation rate in those mutants under submergence. Despite the lower carbohydrate consumption in prt6 mutants (Fig. 4D), the adenylate content after 24 h of submergence was similar in all genotypes (Supplemental Fig. S7), possibly indicating a more efficient energy usage in prt6 mutants or the use of other energy sources.

The mechanism of how plants reduce their sugar consumption rate under oxygen deficiency stress, despite the availability of enzymes and carbohydrates, is still unclear. We hypothesized that prt6 mutants use an as yet unknown mechanism of down-regulation of respiration and sugar consumption at the time when oxygen inside the leaves reaches a certain threshold better than wild-type plants. However, all aerobic respiration rates that were measured after normal growth or treatment with 24 h of submergence or 6 h of prolonged darkness were similar in prt6 mutants and wild types (Fig. 6). Maybe this higher carbohydrate retention is mediated by an, until now, unidentified protein, which is hypoxia inducible and regulated by the N-end rule pathway. It would, therefore, be highly expressed in prt6 mutants and could decrease the carbohydrate consumption rate more than in the wild type if oxygen concentrations fall below a certain point or other starvation signals appear. Indeed, other starvation conditions, such as germination under dark conditions or treatment of seedlings with prolonged darkness, also lead to better survival of prt6 mutants than their respective wild types (Fig. 5). Furthermore, prt6 mutants show reduced root growth at the seedling stage in air that is associated with a reduced breakdown of oil bodies but can be complemented by Suc supplementation (Holman et al., 2009). Therefore, it could be also speculated that an adaptation takes place at the level of energy supply rather than energy consumption. The identification of signals and factors that trigger this response in prt6 mutants would give some unique insights into the regulation of primary metabolism during hypoxia.

CONCLUSION

The identification of a novel mutant allele for PRT6, ged1, reveals a unique link between the N-end rule pathway and resistance to starvation. It could be shown that N-end rule pathway mutants are generally tolerant to submergence-induced starvation as well as starvation in darkness, which was partially associated with carbohydrate conservation under submergence but also, other unidentified factors. This occurred despite the fact that glycolytic and fermentative genes were more highly expressed in these mutants. This suggests that the N-end rule pathway is involved in a quiescence-like response during submergence in Arabidopsis.

MATERIALS AND METHODS

Plant Material

Seeds of the WS ecotype and ged1 were obtained from John C. Gray (Choy et al., 2008). Briefly, ged1 is an EMS mutant of a transgenic plant expressing GFP under control of a tobacco (Nicotiana tabacum) RbcS (Rubisco small subunit) promoter (Choy et al., 2008). The Syngenta Arabidopsis Insertion Library T-DNA insertion line pprt6-1 and the respective Col-0 ecotype were obtained from Julia Bailey-Serres (Gibbs et al., 2011).

Analysis of Gene Expression

Microarray data from selected published experiments were downloaded from the Gene Expression Omnibus database (GSE12401, GSE14578, GSE9719, GSE29800, GSE39384, and GSE28800) and reanalyzed as previously described (Mustroph et al., 2010).

RNA was extracted from 7-d-old Arabidopsis (Arabidopsis thaliana) seedlings using TRIzol (Bioline) according to the manufacturer’s instructions. Subsequently, 1 μg of total RNA was reverse transcribed with oligo(dT) using RevertAid Reverse Transcriptase (Fermentas) and used for semiquantitative PCR analyses.

Map-Based Cloning

Map-based cloning of the ged1 mutation was done on the F2 generation of a cross between ged1 and Col-0. Individual F2 plants were chosen according to their sensitivity of seedling establishment on MS medium containing 1% (w/v) Suc, 1% (w/v) agar, and 0.1 μM ABA. Genomic DNA was extracted from leaf samples after recovery and growth on soil. F3 seeds were harvested to exclude false-positive selections in selected individuals; 26 simple sequence length polymorphism and cleaved amplified polymorphic sequence markers across five Arabidopsis chromosomes were used to map the mutation on a chromosome region. For subsequent fine mapping, several additional primers on chromosomes 3 and 5 were used (Supplemental Table S4).

Because of the lack of recombination events on the affected region on chromosome 3, another strategy was applied to find the mutated gene, namely by crossing of the mutant with already published T-DNA insertion null alleles with the same phenotype (prt6-1 and at1at2; Graciet et al., 2009; Holman et al., 2009; Gibbs et al., 2011; Licausi et al., 2011). The F1 generation of the
crosses was tested for ABA-sensitive germination. After identification of the affected gene as **PRT6**, its genomic sequence was determined from overlap approximately 2,000-bp-long fragments by sequencing of the PCR products. Because one region could not be amplified by PCR in **gol1**, a TAIL PCR was performed from the left and right sites of the region according to the method previously described (Liu et al., 1995). For this, subsequent PCRs with the primers `pren3_3b fw`, `pren3_3d fw`, and `pren3_3c fw` were done (Supplemental Table S4) combined with one of the respective random primer pools: AD1 to AD6 (Mustroph et al., 2009). In the reverse direction, the primers `pren6_rev3`, `pren6_rev3n`, and `pren6_rev3d` were used. Respective PCR products of the third PCR were sequenced.

**Submergence Survival Experiments**

The submergence survival experiments were carried out at the University of Unicamp as described by Vashishth et al. (2011). Briefly, plants were grown on soil until the 10-leaf stage in 9-h-light (200 μmol photons m⁻² s⁻¹)/15-h-dark cycles. Stress treatment was started 2 h after the start of a light cycle; 10 plants per genotype treatment and time point were allowed to recover for 2 weeks in a growth chamber with 70% humidity and light conditions as mentioned above.

For additional survival experiments at Bayreuth University, plants were germinated on MS medium for 1 week and grown on soil for 4 weeks in short-day conditions; subsequently, plants were submerged in tap water at the end of the light period in complete darkness. Five and 7 d later, plants were taken out of the water and directly photographed. For recovery, one-half of the plants were grown for another 14 d at low-humidity levels in the growth chamber (about 20%) in short-day conditions, whereas the other one-half were covered with a light-transmittable lid to increase humidity around the plants to about 100%. Pictures were taken again after 14 d of recovery.

**Plant Growth and Treatment for Sugar Measurements**

Seeds were incubated in deionized water at 4°C for at least 3 d. Several seeds per genotype were sown in the same pot on sterilized soil. In the case of the submergence treatment, a mesh was spanned over the soil to prevent its disturbance during the sinking process. After 1 week, late-germinated seedlings were removed, so that only one plant per pot remained. Plants were grown for 5 weeks in total at 23°C in a growth chamber with 8-h-light (80 μmol photons m⁻² s⁻¹)/16-h-dark cycles. Stress treatments were started at the end of a light cycle. For submergence treatments, three plants per genotype and time point were put in the same box (40-cm length × 31-cm width) filled with acclimated tap water, and glass weights were used to sink the pots. After 8 and 24 h, rosette leaves were detached at the petiole-leaf blade junction, omitting the two oldest and very small young leaves. A separate pool of rosette leaves was created for each plant, the respective fresh weights were measured, and samples were then immediately frozen in liquid nitrogen. Leaves of submerged plants were gently dabbed with tissue paper before weight measurement. The corresponding air-treated plants were processed similarly and grown and stressed together with the plants for the submergence treatment. For anoxia treatments, two pots per genotype were put in a desicciator, in which nitrogen gas was continuously pumped and allowed to exit under positive pressure, thereby replacing the air. Separate air controls with two plants per genotype that were grown and treated together with the plants used in the anoxia experiment were harvested. All experiments were repeated three times.

**Metabolite Extraction and Measurements**

Frozen rosette leaves of each plant were ground, mixed with 0.5 mL of 0.83 N perchloric acid, and centrifuged; the supernatant was isolated and neutralized by the addition of 125 μL of 1 N bicarbonate and 75 μL of 4 μL KOH. After centrifugation, the supernatant was isolated and used for soluble sugar measurements. For starch measurements, the pellet of the first centrifugation step was washed twice with 80% (v/v) ethanol; 400 μL of 0.2 μL KOH was added to the pellet and incubated for 1 h at 95°C. After centrifugation, the supernatant was isolated and neutralized with 80 μL of acetic acid; 50 μL of this extract was mixed with 100 μL of 50 mM sodium acetate buffer, pH 5.0, containing 0.5 units of amyloglucosidase and incubated at 55°C overnight. Sugars were measured with a spectrophotometer at 340 nm based on the conversion of NAD to NADH by Glc-6-P dehydrogenase; 50 μL of extract was mixed with 800 μL of 0.1 μL imidazole buffer, pH 6.9, containing 5 mM MgCl₂, 1 mM ATP, 0.5 μM NAD, and 1 unit of Glc-6-P dehydrogenase. After stabilization of the absorption, 0.5 units of hexokinase, 0.5 units of phosphoglucosamine isomerase, and 60 units of invertase were added in subsequent steps to determine Glc, Fru, and Suc, and the respective changes in absorption were measured. Adenylates (ATP and ADP) were measured as previously described (Mustroph et al., 2006).

**Measurement of Respiration Rates**

For detecting aerobic dark respiration on seedlings, Arabidopsis seeds were surface sterilized by incubation in chlorine gas for 45 min. Seeds were sown on MS medium with 1% (w/v) agarose, pH 5.7, without the addition of Suc and kept at 4°C for 3 d. After 7 d of growth under long-day conditions at 23°C, plants were directly used for the experiments about 6 to 8 h after the start of the day. Another set of seedlings was dark adapted shortly after the start of the day for another 6 to 8 h.

Respiration rates, as the rates of oxygen consumption, were measured from seedlings immersed in tap water with the help of a Clark-type oxygen electrode at a constant temperature of 20°C for about 5 min per plant batch. About 15 to 20 seedlings were used for one batch, and the fresh weight was determined as the basis for calculations.

For measuring respiration of rosette leaves, 5-week-old Arabidopsis plants were used. Seeds were sown on a soil/perlite (1:2) mixture and kept in the dark at 4°C for 4 d. The seeds were then germinated in climate chambers at 20°C with 180 μmol photons m⁻² s⁻¹ (9-h photoperiod) and 70% relative humidity. At the two-leaf stage, individual seedlings were transferred to 70-mL pots and maintained in growth chambers with the same conditions as used for germination. For respiration measurements, eight blocks of plants (each block containing one replica of each accession) were submerged in tap water at 1-h intervals in large 65-L containers at a depth of 10 cm above shoot height in the dark for 24 h (±0.5 h) at 21°C (±1°C).

After 24 h, pots with plants were transferred under water and in dim safe-green light into a water-filled, aluminum foil-wrapped beaker to keep the plants in the dark. A leaf was cut off under water, rapidly transferred into a water-filled glass respiration cuvette (inner volume of 1.5 mL; MiniRespiration System; Unisense), and fixed between two plastic mesh discs above the stirring bar. Then, the cuvette was closed with a glass lid and placed in a thermostat water bath (22°C), and an oxygen microsensor (Unisense) was inserted through a capillary in the lid. After approximately 3 min of acclimation, respiration was measured in the dark as oxygen depletion during 5 to 10 min. Depletion curves were linear and typically started around air saturation (21 kPa of oxygen), with a subsequent decrease of oxygen concentrations over 10 min of less than 1 kPa. We anticipated that oxygen concentrations in leaves of the submerged plants had not reached hypoxic conditions before the measurements (Vashishth et al., 2011). After each measurement, leaves were carefully blotted dry and weighed.

**Survival of Darkness Experiments with Seedlings**

Seeds were surface sterilized by incubation in chlorine gas for 45 min. Seeds were sown on one-half-strength MS medium with 1% (w/v) agarose, pH 5.7 and kept at 4°C for 4 d. For darkness treatments at the seed stage, plates were put into light for 6 to 8 h (at the start of a 16-h-light/8-h-dark cycle) to induce germination. Plants were then grown vertically in darkness for exactly 8 (Col-0 background) and 10 d (WS background) and then, transferred to the same light conditions again for 7 d. For darkness treatments of seedlings, plants were grown under the same light conditions mentioned above for 7 d and transferred to darkness 6 h after the start of the light cycle for 12 d depending on the first sight of yellowing leaves. For recovery, plates were transferred back to the same light conditions for another 7 d.

Arabidopsis accession numbers of the genes studied according to The Arabidopsis Information Resource are as follows: **PRT6**, At5g05700; **ATE2**, At1g05700; **ATE2**, At1g05700; **MYB3R3**, At3g09370. (https://plantphysiol.org)

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Alignment of TAIL PCR results with the genes **PRT6** and **MYB3R3** from the 3’ site.

**Supplemental Figure S2.** Alignment of TAIL PCR results with the genes **PRT6** and **MYB3R3** from the 5’ site.

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Supplemental Figure S3. Alignment of sequencing results for the gene ATE2 from genomic DNA showing a nucleotide insertion.

Supplemental Figure S4. Alignment of sequencing results for the gene ATE2 from complementary DNA showing a nucleotide insertion.

Supplemental Figure S5. Alignment of translated protein sequences for ATE2 from complementary DNA sequences.

Supplemental Figure S6. Submergence survival under different recovery conditions.

Supplemental Figure S7. Adenylate content of different genotypes before and after stress treatment.

Supplemental Figure S8. Survival of seedlings under dark germination with or without Suc addition.

Supplemental Figure S9. Section of an alignment of two syntenic regions on chromosomes 5 and 5 of Arabidopsis.

Supplemental Table S1. Comparison of microarray experiments.

Supplemental Table S2. Overview of available prt6 mutant alleles

Supplemental Table S3. Statistical analysis of sugar content in Arabidopsis plants from Fig. 4.

Supplemental Table S4. Sequence of primers used in this study.

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