Journal of Experimental Botany, Vol. 62, No. 15, pp. 5623–5632, 2011
doi:10.1093/jxb/err251 Advance Access publication 23 August, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Pyrimidine degradation influences germination seedling growth and production of *Arabidopsis* seeds

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Received 18 May 2011; Revised 30 June 2011; Accepted 18 July 2011

Abstract

PYD1 (dihydropyrimidine dehydogenase) initiates the degradation of pyrimidine nucleobases and is located in plastids. In this study, a physiological analysis of PYD1 employing T-DNA knockout mutants and overexpressors was carried out. *PYD1* knockout mutants were restricted in degradation of exogenously provided uracil and accumulated high uracil levels in plant organs throughout development, especially in dry seeds. Moreover, *PYD1* knockout mutants showed delayed germination which was accompanied by low invertase activity and decreased monosaccharide levels. Abscisic acid (ABA) is an important regulator of seed germination, and ABA-responsive genes were deregulated in *PYD1* knockout mutants. Together with an observed increased *PYD1* expression in wild-type seedlings upon ABA treatment, an interference of PYD1 with ABA signalling can be assumed. Constitutive *PYD1* overexpression mutants showed increased growth and higher seed number compared with wild-type and knockout mutant plants. During senescence *PYD1* expression increased to allow uracil catabolism. From this it is concluded that early in development and during seed production PYD1 is needed to balance pyrimidine catabolism versus salvage.

Key words: Arabidopsis, dihydropyrimidine dehydrogenase, Pyd1, pyrimidine, senescence, uracil.

Introduction

Nucleotides are uniquely important since they represent building blocks of genetic information (DNA and RNA), represent major energy carriers, and pyrimidine nucleoside diphosphate sugars are activated intermediates in the synthesis of lipids, sucrose, and cell wall polysaccharides (Moffatt and Ashihara, 2002; Boldt and Zrenner, 2003; Kafer *et al.*, 2004; Zrenner *et al.*, 2006). Furthermore, nucleotides are core elements of cofactors such as NAD, FAD, *S*-adenosylmethionine, or coenzyme A (CoA), which serve in essential biochemical reactions, such as the synthesis of phospholipids and polysaccharides. By reviewing the recently published work on nucleotide metabolism, it becomes obvious that many facets of this important biochemical aspect of plant metabolism are still poorly understood. One possible reason for this is the complexity of a multitude of biochemical reactions that facilitate *de novo* synthesis, degradation, and interconversion (partial degradation and recycling) of nucleotides, nucleosides, and nucleobases. In contrast to nucleotides, nucleosides do not possess phosphate groups, and nucleobases lack the ribose moiety. The recycling of nucleosides and nucleobases is also known as salvage. In the salvage pathway, nucleobases and nucleosides are converted into nucleoside monophosphates by the action of phosphoribosyl-transferase and nucleoside kinase, respectively (Moffat and Ashihara, 2002; Zrenner *et al.*, 2006; Islam *et al.*, 2007; Mainguet *et al.*, 2009). A great benefit of the salvage pathway is the lower energy cost compared with *de novo* synthesis. At3g53900 (UPP) has been described as being responsible for most of the uracil-phosphoribosyl-pyrophosphatase activity, and this enzyme is localized in the plastid (Mainguet *et al.*, 2009). However, previous work contradicts these findings by claiming that At5g40870 (AtUK/UPRT1) localized to the cytosol exhibits

activity for uridine kinase and uracil-phosphoribosyl-pyrophosphatase (Islam *et al.*, 2007).

De novo synthesis leads to the formation of nucleoside monophosphates, in the case of pyrimidine synthesis to produce uridine-5'-monophosphate (UMP) which then can be converted to nucleotide di- and triphosphates (Moffatt and Ashihara, 2002; Stasolla *et al.*, 2003). The recently identified uridine nucleosidase, NSH1, plays a pivotal role in the regulation of nucleoside, mainly uridine, degradation. NSH1 (formerly named URH1) from *Arabidopsis* was characterized by complementation of a yeast mutant and synthesized as a recombinant protein in *Escherichia coli*. The pure recombinant protein exhibits the highest hydrolase activity for uridine, followed by xanthosine and inosine; the corresponding K_m values were 0.8, 1.4, and 0.7 mM, respectively (Jung *et al.*, 2009, 2011). In soluble leaf extracts from *Arabidopsis*, higher affinities for uridine, inosine, and xanthosine hydrolysis were reported (Riegler *et al.*, 2011). The reason for this discrepancy may be the presence of other non-homologous, as yet unknown proteins exhibiting nucleoside hydrolase activity, possibly residing in different subcellular compartments.

The degradation of pyrimidine nucleobase occurs via the 'reductive' pathway in many organisms (Rawls, 2006; Piskur *et al.*, 2007). Uracil or thymine degradation is initiated by dihydrouracil dehydrogenase [DHUDH; also called PYD1 (this name will be used herein), EC 1.3.1.2]. The resulting product of uracil reduction, dihydrouracil (DHU), can be further degraded by dihydropyrimidinase [DHPase; EC 3.5.2.2 (PYD2)] to β-ureidopropionate (*N*-carbamyl-β-alanine), finally leading to the release of β-alanine, CO_2, and NH_3 by β-ureidopropionase [β-UPase; EC 3.5.1.6 (PYD3), Zrenner *et al.* (2009)]. Each of the above-mentioned enzymes is encoded by a single gene (Zrenner *et al.*, 2009). Chemically, pyrimidine catabolism represents a reversion to *de novo* synthesis (Zrenner *et al.*, 2006). Green fluorescent protein (GFP) fusion studies revealed a cytosolic localization for NSH1 (Jung *et al.*, 2009) and β-UPase, localization of PYD1 within the plastids, and of DHPase within the secretory system (Zrenner *et al.*, 2009). The distribution of these enzymes within different subcellular compartments suggests a potentially complex regulation. β-Alanine, one of the products of pyrimidine catabolism, may become incorporated into pantothenate (vitamin B5), which is an essential precursor in CoA synthesis (Coxon, *et al.*, 2005). However, there is experimental evidence that β-alanine from uracil does not contribute significantly to pantothenate biosynthesis in plants (for a discussion, see Zrenner *et al.*, 2009).

Abscisic acid (ABA) is an important player in the early phase of seed germination. ABA inhibits endosperm rupture and seedling growth, but its contents decline sharply during stratification (Weitbrecht *et al.*, 2011). After rehydration, activation of respiration and amino acid synthesis are very early metabolic events, followed by translation from stored RNAs (Weitbrecht *et al.*, 2011). Nucleobase transporters AtUPS1 and AtUPS2 are localized in vascular tissues and may function as uracil transporters in *Arabidopsis*. High expression of *AtUPS1* during the first days of germination may satisfy the initial nucleobase demand of seedlings (Schmidt, *et al.*, 2004; Zrenner, *et al.*, 2009). A major function of the pyrimidine catabolic pathway may be to regulate cellular levels of free pyrimidine nucleotides during growth and development, or in response to nitrogen limitation (Hewitt *et al.*, 2005; Zrenner *et al.*, 2009). The entire pathway recycles pyrimidine nitrogen to general nitrogen metabolism (Zrenner *et al.*, 2009). Genome-wide expression analysis of primary and secondary metabolism in *Arabidopsis* liquid-cultured seedlings confirmed that reductions in nitrogen availability lead to increased *PYD1* expression which can be rapidly reversed by the addition of nitrate to the growth medium (Scheible *et al.*, 2004). In this study, a physiological analysis of the catabolic pathway enzyme PYD1 employing T-DNA knockout mutants and plants with increased activity of this enzyme was carried out. Evidence is provided that PYD1's action is of major importance for proper seed germination and seed production. Moreover a function in recycling of pyrimidine nitrogen to general nitrogen metabolism during senescence is further substantiated.

Materials and methods

Plant material and growth conditions

Wild-type and transgenic *Arabidopsis thaliana* (L.) Heynh. plants (ecotype Columbia) were used throughout. Prior to germination, seeds were incubated for 24 h in the dark at 4 °C for imbibition, unless stated otherwise in standardized ED73 soil (Weigel and Glazebrook, 2002). Plant growth was carried out at 22 °C and 120 μmol quanta m^{-2} s^{-1} in a 10 h light/14 h dark regime in a growth chamber. As the light source, fluorecence tubes (Osram lumilux cool white and warm white alternating) were used. For growth experiments on sterile agar plates, surface-sterilized seeds were sown on Murashige and Skoog (MS) medium, supplemented with the indicated nucleoside analogues as described previously (Reiser *et al.*, 2004). Growth of plants in liquid culture was performed as described in Scheible *et al.* (2004) with the modification that growth proceeded under the light regime given above.

In addition to wild-type plants, the following T-DNA insertion mutants were used: SAIL_363_E04 (designated *PYD1-1*; Sessions *et al.*, 2002) and GK-251F09 (designated *PYD1-2*; Rosso *et al.*, 2003) obtained from the NASC (National Arabidopsis Stock Center; Scholl *et al.*, 2000)

Generation of mutants

To generate mutants expressing *PYD1* under control of the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter, the complete coding region of *PYD1* was initially amplified by PCR from *Arabidopsis* leaf cDNA using the primers mentioned in Supplementary Table S1 available at *JXB* online, and inserted into an *Eco*RV-cleaved vector (pBluescript, Stratagene, Heidelberg). The complete coding region was then excised from this vector with *Eco*RI/*Cla*I and inserted into pHannibal (Wesley *et al.*, 2001) via the same restriction sites. The resulting pHannibal constructs were subcloned into the binary vector pART27 as detailed in Reiser *et al.* (2004). Subsequently, the plasmids were used for *Agrobacterium tumefaciens* transformation. Transformation of *Arabidopsis* was performed according to the floral-dip method (Clough and Bent, 1998).

Germination experiments

Endosperm rupture (germination) was monitored as described in Müller at al. (2006). Fifty seeds (9 months after harvest) were put on 0.1-fold strength MS agar plates and incubated under constant illumination (10 µmol quanta m^{-2} s^{-1}) with white fluorescent tubes at 24 °C. Plates with seeds were stratified for 48 h at 4 °C in the dark when indicated. After the indicated time periods, endosperm rupture was monitored under a stereomicroscope (Leica MZ12, Leica, Germany).

Quantitative RT-PCR

Quantitative reverse transcription-PCR (RT-PCR) was performed as described in Leroch *et al.* (2005). Gene-specific primers used are listed in Supplementary Table S1. The gene At1g07930 encoding elongation factor 1α (EF1α) was used for quantitative normalization (Curie *et al.*, 1991).

Extraction of total RNA and RNA gel-blot hybridization

Total RNA was isolated from frozen tissue samples by using the Purescript RNA extraction kit (Gentra Systems) according to the manufacturer's instructions. RNA samples (10 µg) were denatured and separated on a 1.2% agarose/2.5% formaldehyde gel. Ethidium bromide was included in the loading buffer medium to confirm equal sample loading. Isolated RNA was blotted onto a nylon membrane (Nytran-Plus, Schleicher & Schüll, Dassel, Germany), and *PYD1*, restricted from the corresponding pBluescript construct with *Eco*RI/*Cla*I, was ^{32}P-labelled by random priming (Ready-To-Go DNA labeling beads, Amersham Pharmacia). Prehybridization and hybridization were performed at 65 °C in Church buffer. The final blots were visualized by a PhosphorImager (Packard, Dreieich, Germany).

Quantitation of [^{14}C]pyrimidine degradation

For analysis of the [^{14}C]nucleoside or nucleobase degradation, given as [^{14}C]CO_2 liberation, 7-day-old *Arabidopsis* seedlings grown in hydroponic culture were used (Scheible *et al.*, 2004). Three seedlings were incubated in 2 ml reaction tubes with 800 µl of 5 µM of the indicated, ^{14}C-labelled pyrimidines (1 GBq $mmol^{-1}$) in growth media. On the inside at the top of this tube, a small 0.5 ml reaction vessel containing 100 µl of 1 M KOH was fixed with grease to allow [^{14}C]CO_2 absorption. The seedlings were allowed to float on the solution, and incubation was continued for 24 h in the dark. The reaction was stopped by adding 100 µl of 2 M HCl with a syringe through the closed lid. The hole in the lid was sealed with grease and, after subsequent incubation for 12 h, the released radioactively labelled [^{14}C]CO_2 was quantified in a scintillation counter.

Determination of metabolite contents and enzyme activities

Sugar quantification in fractionated samples was conducted by ion chromatography on an HPLC CarboPac PA1 column (Dionex) followed by amperometric quantitation as described (Zuther *et al.*, 2004). Amino acids were quantified as described in Jung *et al.* (2009), and starch was quantified according to Reinhold *et al.* (2007). For the determination of protein and lipids in seeds, the method of Reiser *et al.* (2004) was applied. Uracil was determined using the same method as used to determine uridine (Jung *et al.*, 2009). The relative contents of total carbon and total nitrogen were determined in dry powdered samples with an elemental analyser (Vario EL II, Elementaranalysensysteme GmbH, Hanau, Germany) according to the manufacturer's instructions.

Invertase activity was quantified spectrophotometically in a coupled enzyme assay at pH 7.4 after extraction in HEPES/KOH pH 7.4 (50 mM), $MgCl_2$ (5 mM), EDTA (1 mM), and phenylmethylsulphonyl fluoride (PMSF; 0.1 mM).

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) with Dunnett's post-test compared with the corresponding wild type. The analysis software was embedded in the GraphPad Prism software 5 (GraphPad software Inc.). *P*-values are indicated by asterisks (*P <0.05, **P <0.01, ***P <0.005).

Results and Discussion

Analysis of PYD1 expression and biochemical function using mutants

PYD1 (At3g17810) was recently shown to catalyse the conversion of uracil to dihydrouracil in *Arabidopsis*. This was achieved by the use of corresponding T-DNA insertion mutants which showed lowered catabolism of exogenously supplied uracil (Zrenner *et al.*, 2009). In the analysis presented here, the results of Zrenner *et al.* (2009) are supported and a comprehensive physiological analysis highlighting an important function of PYD1 during early and late *Arabidopsis* development is presented. As a starting point, the expression of PYD1 was monitored by quantitative RT-PCR in *Arabidopsis* tissues from soil-grown plants. Whereas PYD1 expression was quite similar in roots, 4- and 8-week-old leaves, stems, and siliques, a 2-fold higher expression was seen in flowers (Fig. 1). It was striking to see a very marked expression in dry seeds which was ~28 times higher compared with roots or leaves (Fig. 1). Microarray data support this expression profile (Zimmermann *et al.*, 2004). Further examples of the accumulation of translatable RNAs in dry seeds are LEA (late embryo abundant) mRNA, and mRNAs for seed storage proteins and for heat shock factors. In addition, transcripts from genes encoding enzymes of metabolism such as an aquaporin, isocitrate lyase, and malate synthase have been found (Kimura and Nambara, 2010). For the further analysis of PYD1 function, T-DNA insertion mutants were analysed and, in addition constitutive CaMV-35S overexpression lines (*35S:PYD1*) were generated.

Two knockout lines were analysed, GK-251F09 (designated *PYD1-1*) and SAIL_363_E04 (designated *PYD1-3*;

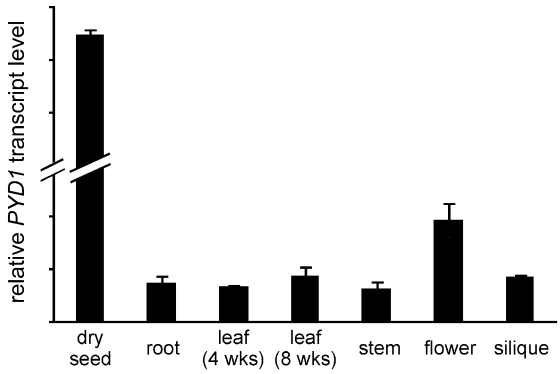

Fig. 1. Relative expression of *PYD1* in *Arabidopsis* tissues. *PYD1* expression was monitored by quantitative RT-PCR using EF1α as the reference gene.

Supplementary Fig. S1A at *JXB* online). The former line has been described as a *PYD1* knockout previously (Zrenner *et al.*, 2009). First, the presence of a T-DNA insertion in both mutants was checked by PCRs with *PYD1*-specific primers (*PYD1* KO fwd/rev) and T-DNA-specific primers (SAIL_LB for *PYD1-3* and GK_LB for *PYD1-1*) (Supplementary Fig. S1B, C). Secondly, the absence of an amplification product with gene-specific primers on genomic DNA in homozygous plants was checked (Supplementary Fig. S1B) and, furthermore, the lack of a gene-specific PCR product in cDNA preparations from both *PYD1-1* and *PYD1-3* mutants was confirmed using primers PYD1_fw and PYD1_rev (Supplementary Fig. S1D). As a control, cDNA prepared from wild-type plants gave rise to a PCR band of the expected size of 1281 bp (Supplementary Fig. S1D).

Three independent, constitutive overexpression lines (*35S:PYD1*) were also generated, as described in the Materials and methods. All three lines accumulated significantly increased transcript levels as compared with the wild type (Fig. 2A). Specifically, plants from lines #24, #11, and #19 showed 74-, 95-, and 156-fold increased transcript levels, respectively (Fig. 2B). As no direct enzyme activity assay is available for PYD1 (for discussion, see Zrenner *et al.*, 2009), changes in activity of all mutants were monitored by the release of radiocarbon ($[^{14}C]CO_2$) after feeding [^{14}C]uridine, [^{14}C]uracil, or [^{14}C]dihydrouracil to 10-day-old liquid-grown seedlings (Fig. 2C–E). The CO_2 release by wild-type plants from uridine, uracil, and dihydrouracil accounted for 28, 22, and 49% of the imported metabolite, respectively. In contrast, *PYD1-1* and *PYD1-3* knockout lines released only 3% and 1% of imported uridine, and 2% and 1% of imported uracil (Fig. 2C–E). In contrast, the CO_2 release by overexpressors from uridine was 2-fold and from uracil 2.5 fold higher compared with the wild-type situation (Fig. 2C, D). No change was observed in the degradation of dihydrouracil in a comparison of all mutants (Fig. 2E). In Zrenner *et al.* (2009) the effect of PYD1 inactivation in corresponding knockout mutants was less drastic. Whereas in that work wild-type seedlings liberated 48% of incorporated uracil as CO_2, the mutants still liberated 29%. The higher uracil catabolism observed may be due to the higher uracil concentration of 2 mM that was applied (Zrenner *et al.*, 2009) compared with 5 μM used here. A higher external concentration will affect the internal concentration and drive more uracil towards catabolism, even in the *PYD1* knockout, indicating the presence of one or more further enzymes exhibiting DHPDH activity. The lower concentration of 5 μM uracil was used as this fits to the observed affinities of the known plasma membrane uracil transporters UPS1 and UPS2 of ~6 μM (Schmidt *et al.*, 2004). Nevertheless it is not known whether other transport proteins capable of uracil import into cells exist. Nevertheless, these results indicate that PYD1 exerts marked control over uracil degradation. This is further illustrated by the marked susceptibility of both *PYD1:T-DNA* insertion lines towards 100 μM toxic flurouracil (Supplementary Fig. S2A at *JXB* online). In contrast, *PYD1* overexpressors tolerate higher concentrations (200 μM and 500 μM) compared with wild-type seedlings (Supplementary Fig. S2B). This indicates that PYD1 activity is limiting for the detoxification of flurouracil under the experimental conditions used. The observed increased degradation of uridine in overexpressor lines (Fig. 2C) further indicates that the upstream reaction catalysed by NSH1 (formerly known as URH1; Jung *et al.*, 2009, 2011) is accelerated. Also the reciprocal effect (acceleration of pyrimidine catabolism by overexpression of NSH1 (Jung. *et al.*, 2009) was observed. This may be due to changed uracil concentrations or point to a concerted regulation of both NSH1 and PYD1, activity. In addition to uracil, degradation of the only other pyrimidine nucleobase occurring in plants, thymine, is also markedly impaired in *PYD1* knockouts but increased in *PYD1* overexpressor lines (Supplementary Fig. S3).

These results show that PYD1 is crucial for pyrimidine degradation. A localization of PYD1 in plastids was demonstrated by use of a GFP fusion protein [Zrenner *et al.*, 2009; and supported by our own analysis using a similar approach (not shown)]. Evidence for a plastid localization of the main uracil salvage activity has also been presented (Mainguet *et al.*, 2009). Therefore, it appears that the

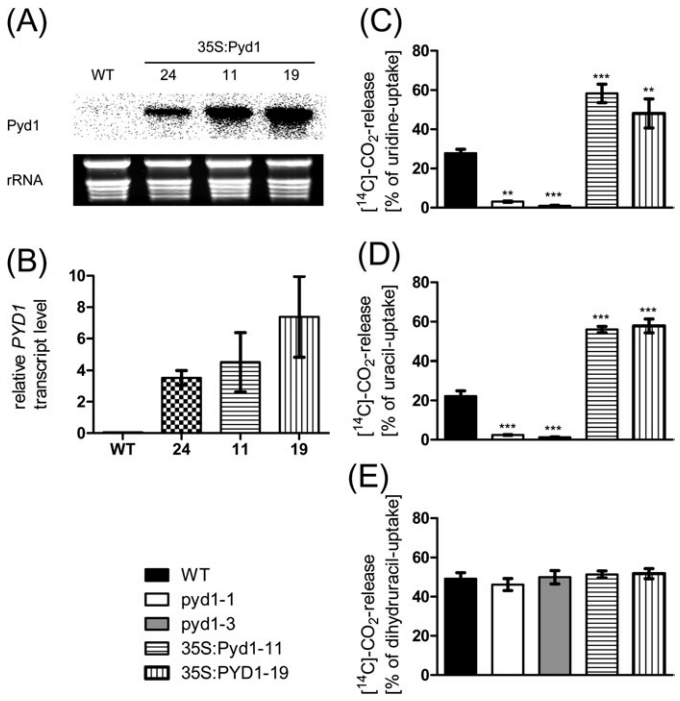

Fig. 2. *PYD1* transcript accumulation in overexpression mutants (*35S:PYD1*) and pyrimidine degradation in *PYD1* knockout and overexpression lines. (A) Expression analysis of *PYD1* overexpression mutants by northern blot hybridization and (B) quantitative RT-PCR. Values represent means ±SE of three biological replicates. (C–E) Catabolism of ^{14}C-labelled uridine, uracil, and dihydrouracil in *PYD1* mutants. Degradation was measured as released $[^{14}C]CO_2$ based on import of the corresponding substrate. Values represent means ±SE of at least five biological replicates. The asterisks indicate significant differences between the wild type and mutants, based on a one-way ANOVA test.

plastid stroma is the site where both uracil degradation and salvage are located. It was supposed that PYD1 from plants lacks an N-terminal cofactor-binding site (Zrenner *et al.*, 2009). Therfore, interaction with a component complementing this lack which must be present in the plastid is required. To test this hypothesis, a 21 amino acid N-terminal truncated PYD1 protein was expressed in *PYD1-3* and found to reside in the cytosol because of lack of a plastid target sequence. The corresponding plants exhibited no PYD1 activity (not shown), supporting the idea that a plastidic cofactor is required for activity. Although experimental evidence for N-terminal processing of PYD1 after import into the plastid which then leads to a truncation of the protein covering ⩾21 amino acids is lacking, it appears less probable that important structural information for the mature protein is present in this region. Furthermore, based on sequence analysis programs for conserved domains (CCDs; Marchler-Bauer *et al.*, 2007), no amino acids involved in cofactor binding in mammalian homologues are present at the far N-terminal part of the proteins.

PYD1 function in seeds and seed germination

Because of the high PYD1 transcript level in dry seeds, the first 3 d of seed germination after imbibition were monitored. For this, seeds were sown on soil or on wet paper. Under both conditions, a delay in seedling development of ~2 d became obvious for both *PYD1:T-DNA* insertion lines (Fig. 3A, B). In contrast, *35S:PYD1* lines grew faster compared with the wild-type controls (Fig. 3A, B). These differences in growth continued subsequently, and were still visible after 6 weeks (Fig. 3C). Flowering also occurred first in the overexpressor lines, followed by the wild type and knockout mutants (Fig. 3D). Thus, a delay throughout the whole plant development of *PYD1* knockout lines has to be attested, whereas overexpressor lines showed a faster development. After 10 weeks of growth, wild-type plants developed 780 siliques with 32 seeds each (mean of at least 10 plants, Fig. 3E–G). Under the same growth conditions *PYD1:T-DNA* mutants developed only 26 and 22 seeds per silique and 629 and 686 siliques per plant, respectively. Overexpressor mutants developed significantly more siliques than wild-type plants. However, the number of seeds per silique was not different from the wild-type situation (Fig. 3E–G).

As the next step, germination itself, which begins with water uptake of the seed and ends when the radicle has protruded through all covering layers (Müller *et al.*, 2006), was looked at more closely. Successful germination can be monitored as endosperm rupture. Endosperm rupture was followed over time for the wild type and *PYD1* mutants with and without prior stratification, as described in Müller *et al.* (2006).

Differences in the germination behaviour between the various *PYD1* mutants became obvious. Whereas wild-type and *35S:PYD1* overexpressors showed 50% germinated

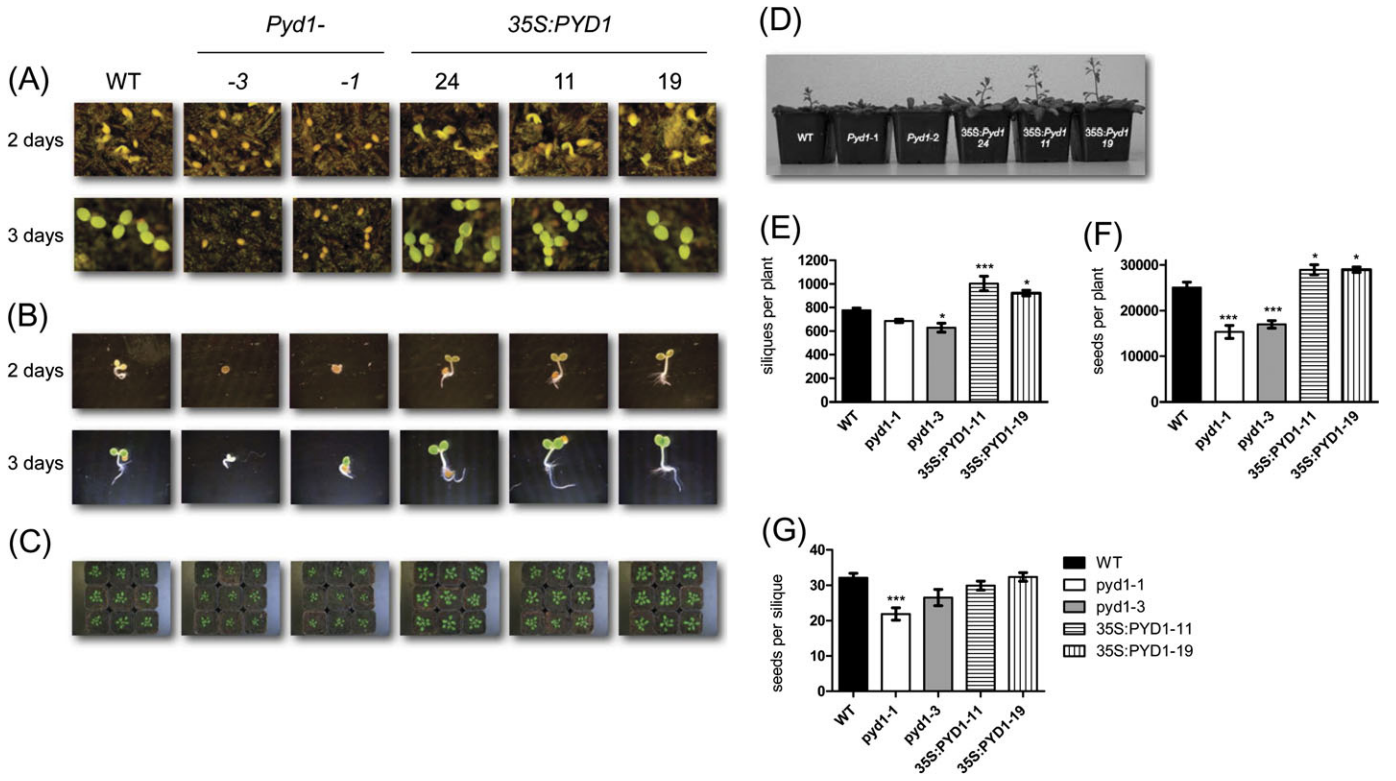

Fig. 3. Growth analysis of wild type (WT), *PYD1* knockout, and *PYD1* overexpressor plants. (A) Germination was monitored on soil after 2 d and 3 d (B) Germination was monitored for 2 d and 3 d on wet paper. (C) Six-week-old plants grown on soil. (D) Typical appearance of *PYD1* mutants at the time of bolting. (E–G) Seeds and siliques were harvested from plants grown for 4 weeks under short day conditions and subsequently for 6 weeks under long day conditions. Values represent means ±SE of at least 10 biological replicates. The asterisks indicate significant differences between the WT and mutants, based on a one-way ANOVA test.

seeds after ~33 h without stratification and after ~22 h with stratification, both knockout mutants germinated markedly more slowly (Fig. 4A, B). *PYD1-1* mutants showed 50% germinated seeds after 48 h without stratification and after 43 h with stratification (Fig. 4A, B). Even after 49 h the *PYD1-3* mutants germinated to 15% without stratification and 37% with stratification (Fig. 4A B). The seeds used were 9 months after harvest, and yet there was still a small effect of stratification in all populations. However, this is typical of other observations on fully after-ripened seeds (Müller *et al.*, 2006). The wild type and *PYD1* overexpressor mutants showed no difference in endosperm rupture, thus it can be concluded that in this case post-germination effects account for the faster development of the corresponding plants. In contrast, *PYD1-1* and *PYD1-3* mutants were markedly delayed in germination. ABA is known to act in an inhibitory manner on dormancy and thus seed germination, while GA (gibberellic acid) is an antagonist of ABA. A current model of how endosperm rupture (seed germination) is regulated is presented in Linkies *et al.* (2009). While water uptake and testa rupture are not affected by ABA, the procedure of endosperm cap weakening, a prerequisite of endosperm rupture, is inhibited by ABA. GA, as an embryo signal, can induce the cap weakening process. In the late phase of germination, ethylene biosynthesis comes into play and interferes with ABA signalling (Linkies *et al.*, 2009).

Therefore, a possible role for ABA in the delay of germination of *PYD1* knockout mutants was analysed. When 9-day-old seedlings grown in liquid culture were subjected to ABA treatment, a 3- to 6-fold increase in *PYD1* transcript was observed, dependent on the ABA concentration (Fig. 5A). These results are in accordance with data available through the eFP browser database (Winter *et al.*, 2007). This pointed to an interaction of PYD1 function and ABA metabolism. To proceed further in this analysis, ABA-responsive genes were analysed with respect to their expression in the *PYD1:T-DNA* mutants. ABI1 is known to be a regulator of ABA signalling during germination and shows increased transcript levels as a response to ABA treatment (Hoth *et al.*, 2002). *PYD1:T-DNA* seeds, 2 d after imbibition, showed a 5-fold increased ABI1 expression compared with wild-type seeds of the same age (Fig. 5B). As a further marker for the ABA status, ABF4 was monitored. ABF4 is an ABA-responsive element-binding factor and member of the bZIP transcription factors, up-regulated by ABA (Choi *et al.*, 2000). ABF4 showed the opposite response compared with ABI1 in the

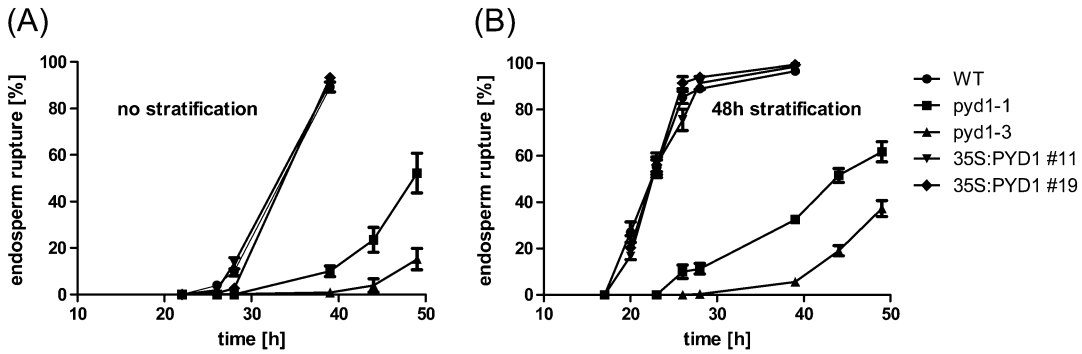

Fig. 4. Germination of seeds was monitored and shown as endosperm rupture. (A) Seeds were incubated for the indicated times and monitored for endosperm rupture without stratification. (B) Seeds were stratified for 48 h in the dark at 4 °C. Five plates with 50 seeds each were analysed and the mean values ±SE were calculated.

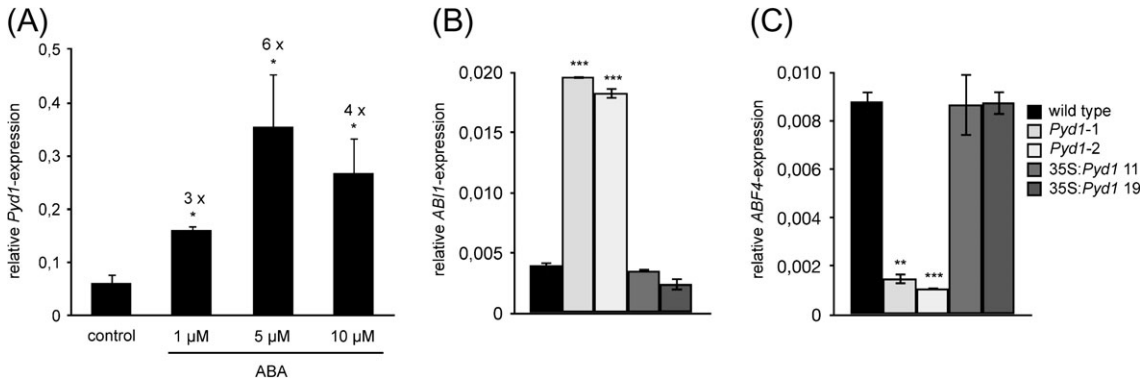

Fig. 5. Relative transcript level of *PYD1* in response to ABA treatment and relative expression of ABI and ABF4 in 2-day-old *PYD1* mutants. Expression was monitored by quantitative RT-PCR using EF1α as the reference gene. Values represent means ±SE of at least three biological replicates. The asterisks indicate significant differences between the wild type and mutants, based on a one-way ANOVA test.

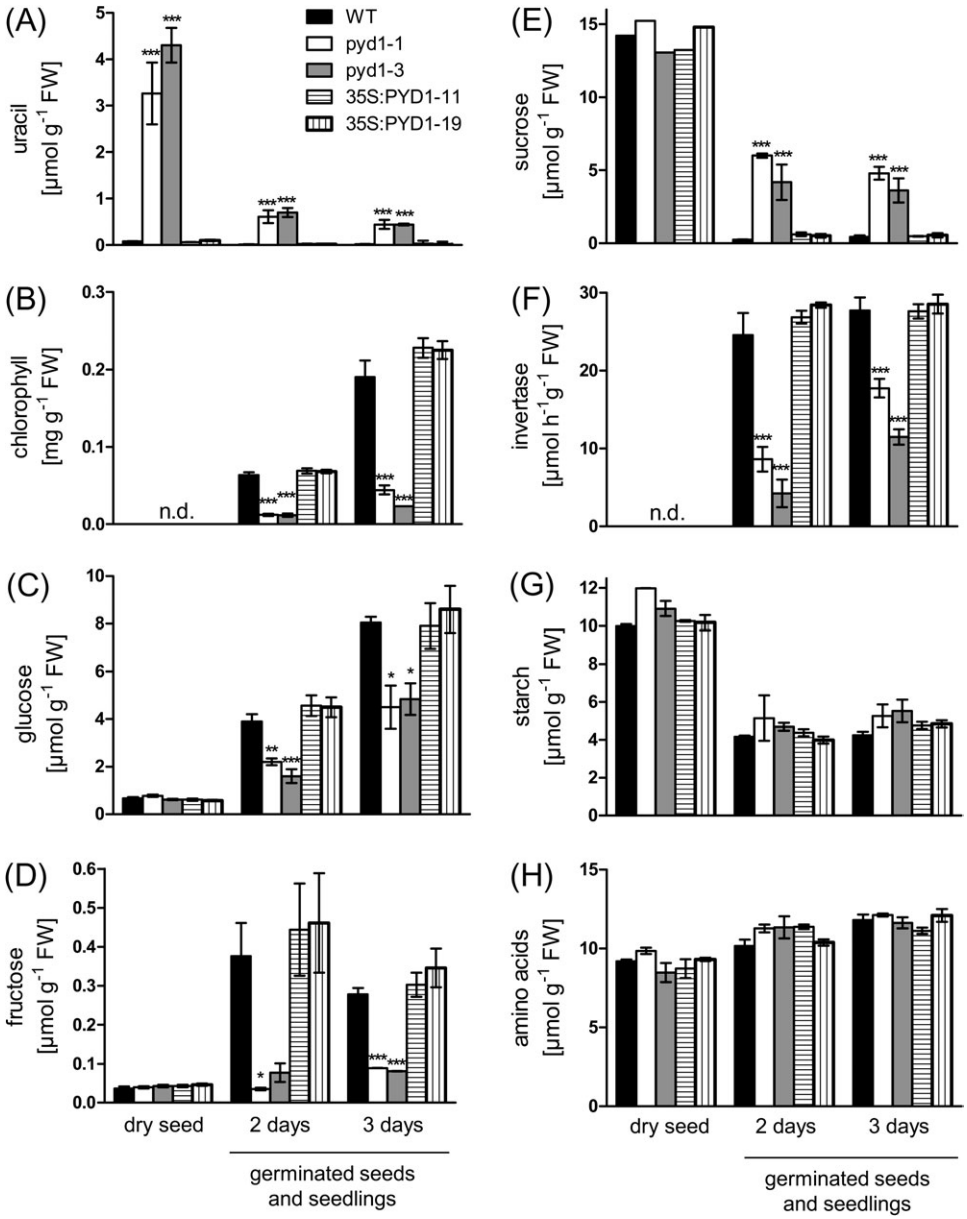

Fig. 6. Metabolite levels and invertase activity (as indicated) determined in dry seeds, and 2- and 3-day-old seedlings. Seeds were sown on wet paper in trays and incubated for 48 h in the dark at 4 °C for imbibition. The trays were then transferred to a growth chamber (see Materials and methods) and plant material was harvested after 48 h or 72 h (4 h after lights on). Values represent means ±SE of at least five biological replicates. The asterisks indicate significant differences between the wild type and mutants, based on a one-way ANOVA test.

present experiments; the relative expression in *PYD1:T-DNA* mutants accounted for 15% of the wild-type level (Fig. 5C). Although the differential expression pattern of ABI1 and ABF4 in the *PYD1* knockout mutants cannot be explained, the fact that both are deregulated and the observed delayed germination point to an interaction of PYD1 with ABA signalling during germination.

To exclude the possibility that an altered composition of seed storage products might be responsible for the observed differences in germination and development of the mutants, total carbon and nitrogen in dry seeds was determined by elemental analysis and, in addition, total protein and lipid were extracted and quantified from seeds. No significant differences were detectable between all mutants and wild-type plants with respect to all storage compounds (Supplementary Table S2 at *JXB* online). Obviously, PYD1 activity does not influence seed development with respect to the main storage compounds but acts from the time of germination onwards until new seed is produced.

Due to the limited ability of *PYD1* knockout lines to degrade uracil, markedly increased uracil levels up to 50-fold compared with the wild type were found in dry seeds of *PYD1* knockouts (Fig. 5A). The uracil level remains high during the first days of germination, although the amount on a fresh weight basis declines as a consequence of hydration and cell expansion of the seeds (Fig. 6A). Also

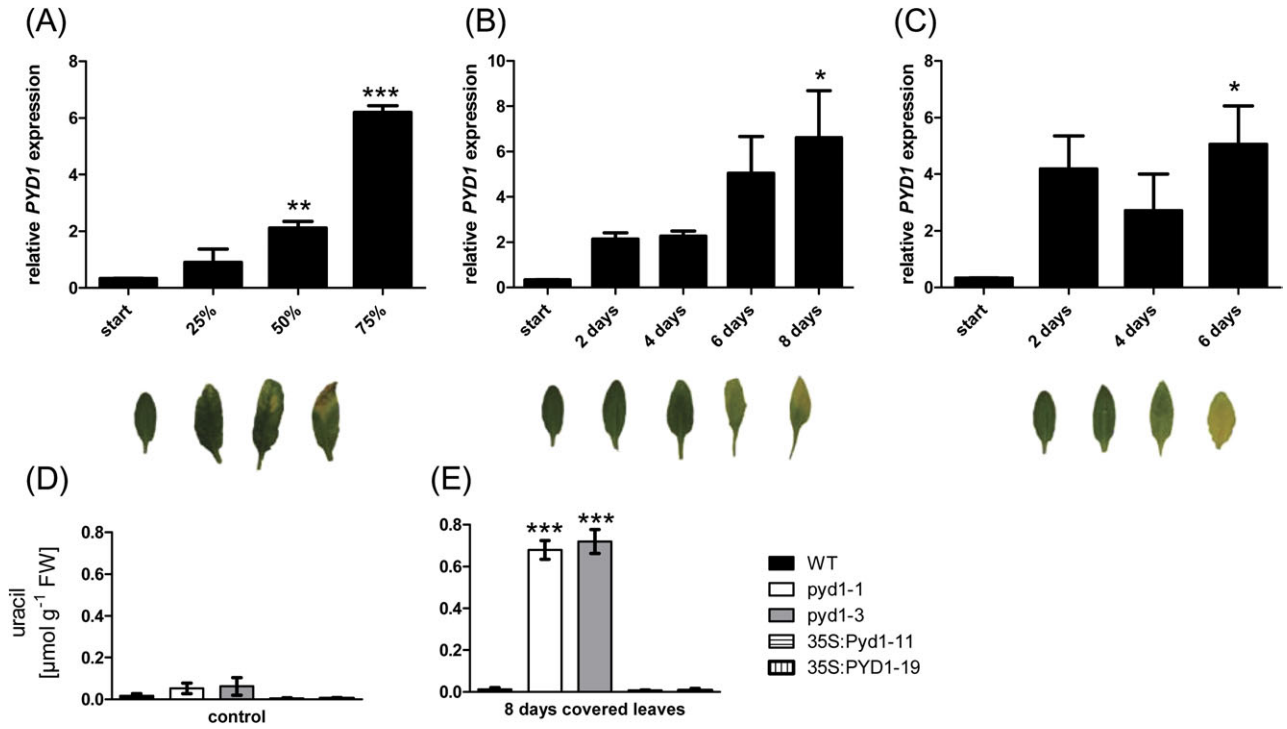

Fig. 7. Relative *PYD1* transcript accumulation monitored at different stages of leaf sensescence and uracil levels in senescent leaves from *PYD1* mutants. (A) Samples were taken from plants undergoing natural senescence, (B) plants with covered leaves, and (C) detached leaves. Expression was monitored by quantitative RT-PCR using EF1α as the reference gene. (D) Uracil was determined in leaves covered for 8 d. Values represent means ±SE of at least three biological replicates. The asterisks indicate significant differences between the wild type and mutants, based on a one-way ANOVA test

other plant tissues such as senescent leaves, stems, and siliques are characterized by markedly increased uracil levels (Supplementary Fig. S4A–C). As already indicated (see Fig. 3), development of *PYD1:T-DNA* lines is delayed, which is illustrated by the markedly lowered chlorophyll contents during the first 3 d after imbibition (Fig. 6B). A deeper analysis of the metabolic changes during early germination reveals lower monosaccharide contents in *T-DNA* mutants, accompanied by higher sucrose contents, compared with the wild type, whereas overexpressors show no significant differences (Fig. 6C–E). Sucrose and monosaccharides are connected by the activity of neutral invertase and it was shown that this enzyme activity is indispensable for normal growth of *Arabidopsis* (Barratt *et al.*, 2009). Neutral invertase activity accounted for 24 µmol g FW^{-1} h^{-1} in 2-day-old seedlings of the wild type and had similar activity in 3-day-old seedlings (Fig. 6F). In contrast, the invertase activity in *PYD1-1* and *PYD1-2* was reduced to ~25% in 2-day-old and ~50% in 3-day-old seedlings compared with the wild type (the activity in overexpressor mutants was similar to that in the wild type; Fig. 6F). The amounts ofig. 6G, H). Therefore, it appears that invertase activity is a key regulator of growth affected by PYD1 during the early phase of *Arabidopsis* development.

Is the uracil level the critical factor for the observed delay in development? To test this hypothesis, *Arabidopsis* seedlings were grown on agar plates in the presence of up to 10 mM uracil without observing any growth phenotype. However, it is possible that this experiment does not resemble the situation in *PYD1:T-DNA* mutants as nothing is known about the cell type-specific or subcellular accumulation of uracil in these plants.

The complete degradation of uracil leads to the formation of ammonia and β-alanine. Whereas release of ammonia due to pyrimidine degradation increases under conditions of nitrogen limitation and thus attenuates the effects of nitrogen starvation (Zrenner *et al.*, 2009; own unpublished observations), much less is known about the role of β-alanine in plant metabolism. However, β-alanine represents a precursor of CoA biosynthesis and one prominent biosynthetic reaction requiring CoA is fatty acid synthesis. However, a major contribution of uracil catabolism to pantothenate synthesis is unlikely based on incorporation studies with radiolabelled uracil (for discussion, see Zrenner *et al.*, 2009).

PYD1 function in senescence

At the end of their development, plants enter a programmed ageing process known as senescence. This has been well studied especially in leaves. For this kind of analysis, different experimental approaches are used. Leaves of different ages can be harvested from 'old' plants (natural senescence), leaves can be covered to induce senescence (dark-induced senescence), or leaves can be detached and incubated in the dark (van der Graaff *et al.*, 2006). Under all

conditions described, a marked increase of PYD1 transcript up to 20-fold above controls was observed (Fig. 7A–C). In line with these observations was an increase in uracil accumulation in leaves from *PYD1-1* and *PYD1-2* plants after covering the leaves for 8 d (Fig. 7D). The already elevated uracil content of untreated *PYD1-1* and *PYD1-2* mutant leaves compared with those of the wild type was further increased after covering leaves up to 0.77 and 0.67 μmol g FW^{-1}, respectively. Darkened leaves of wild-type plants still only accumulated 0.02 μmol uracil (Fig. 7D). This is indicative of an up-regulation of pyrimidine degradation in senescent tissues to liberate nitrogen bound in nucleic acids and nucleotides and make these available for other tissues, such as developing seeds. It has been shown that accelerated nitrogen remobilization from senescing leaves, achieved by overexpression of pyruvate phosphate dikinase, positively affected seed weight (Taylor *et al.*, 2010). Furthermore, nitrogen limitation is a condition in which uracil degradation is markedly elevated (Zrenner *et al.*, 2009). Therefore, it is likely that altering the efficiency of uracil catabolism affects seed yield (Fig. 3F) through altered nitrogen supply.

In summary, a delayed germination, seedling development, and altered seed production were observed when PYD1 is missing from *Arabidopsis* plants. One of the earliest metabolic alterations in these mutants is an accumulation of sucrose due to decreased cytosolic invertase activity, in addition to constitutively high uracil levels. The delayed endosperm rupture and altered expression of ABA-dependent genes point to an interaction of PYD1 with ABA signalling which might also influence invertase activity directly. In the later phase of development, PYD1 is involved in nucleic acid breakdown and possibly in remobilization of nitrogen supply to developing seeds.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Genetic characterization of *PYD1* T-DNA insertion mutants.

Figure S2. Growth of the wild type and *PYD1* mutants in the presence and absence of toxic 5-fluorouracil.

Figure S3. Catabolism of ^{14}C-labelled thymine in *PYD1* mutants.

Figure S4. Uracil contents of the wild-type and *PYD1* mutants.

Table S1. PCR primers used in this study.

Table S2. Contents of storage compounds in wild-type and *PYD1* mutant seeds.

Acknowledgements

This work was supported by DFG-grant MO 1032/3-1. We gratefully acknowledge support of this work by Professor H. E. Neuhaus.

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