Proanthocyanidin oxidation of Arabidopsis seeds is altered in mutant of the high-affinity nitrate transporter NRT2.7

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Abstract

NRT2.7 is a seed-specific high-affinity nitrate transporter controlling nitrate content in Arabidopsis mature seeds. The objective of this work was to analyse further the consequences of the nrt2.7 mutation for the seed metabolism. This work describes a new phenotype for the nrt2.7-2 mutant allele in the Wassilewskija accession, which exhibited a distinctive pale-brown seed coat that is usually associated with a defect in flavonoid oxidation. Indeed, this phenotype resembled those of tt10 mutant seeds defective in the laccase-like enzyme TT10/LAC15, which is involved in the oxidative polymerization of flavonoids such as the proantocyanidins (PAs) (i.e. epicatechin monomers and PA oligomers) and flavonol glycosides. nrt2.7-2 and tt10-2 mutant seeds displayed the same higher accumulation of PAs, but were partially distinct, since flavonol glycoside accumulation was not affected in the nrt2.7-2 seeds. Moreover, measurement of in situ laccase activity excluded a possibility of the nrt2.7-2 mutation affecting the TT10 enzymic activity at the early stage of seed development. Functional complementation of the nrt2.7-2 mutant by overexpression of a full-length NRT2.7 cDNA clearly demonstrated the link between the nrt2.7 mutation and the PA phenotype. However, the PA-related phenotype of nrt2.7-2 seeds was not strictly correlated to the nitrate content of seeds. No correlation was observed when nitrate was lowered in seeds due to limited nitrate nutrition of plants or to lower nitrate storage capacity in leaves of clca mutants deficient in the vacuolar anionic channel CLCa. All together, the results highlight a hitherto-unknown function of NRT2.7 in PA accumulation/oxidation.

Key words: Flavonoids, laccase, nitrate, NRT2.7, proanthocyanidins, seeds, transporter, TT1.0.

Introduction

Seed development and maturation lead to accumulation of N and C compounds in embryo such as reserve proteins, lipids, and carbohydrates, which are then used as energy sources during germination. The N compounds accumulated in seeds originate from nitrate (NO₃⁻), amino acids, and peptides transferred from vegetative organs and subsequent synthesis of storage proteins. NO₃⁻ uptake by the roots and its translocation to the aerial part and to the seeds are achieved by transporters of high-affinity and low-affinity systems (reviewed in Dechorgnat et al., 2011). The high-affinity system is ensured by some members of the NRT2 family (seven members) and the low-affinity system by some members of the NRT1 family (or NPF according to the unified nomenclature proposed by Léran et al., 2013; 54 members), which transport also dipeptides (reviewed in Tsay et al., 2007) and other compounds such as auxin, abscisic acid, and glucosinolates (Krouk et al., 2010; Kanno et al., 2012;
Nour-Eldin et al., 2012). \(\text{NO}_3^-\) uptake by roots is mediated mainly by NRT2.1 and NRT1.1 (AtNPF6.3), depending on the \(\text{NO}_3^-\) concentration of the soil solution (below or above 1 mM, respectively). At extremely low \(\text{NO}_3^-\) concentration (below 0.025 mM), NRT2.4 is also active for \(\text{NO}_3^-\) uptake by roots (Kiba et al., 2012). Then, root xylem loading is due to NRT1.5 (AtNPF7.3) (Lin et al., 2008) and root phloem loading to NRT1.9 (AtNPF2.9) (Wang and Tsay, 2011). In shoots, xylem unloading is performed by NRT1.8 (AtNPF7.2) and NRT1.4 (AtNPF6.2) (Chiu et al., 2004; Li et al., 2010). In leaves, up to 50% \(\text{NO}_3^-\) storage is achieved by an anion channel/transporter (chloride channel a, CLCa), which is a nitrate/proton antiporter localized in the tonoplast of foliar cells (Monachello et al., 2009). Regard the travel of \(\text{NO}_3^-\) through the plant, NRT1.7 (AtNPF2.13) has been suggested as an actor in the apoplastic loading of \(\text{NO}_3^-\) into the phloem sap of older leaves (Fan et al., 2009). There, the delivery of \(\text{NO}_3^-\) to the developing seeds is due to NRT1.6 (AtNPF2.12) located in the vacuolar tissue of the silique and funiculus (Almagro et al., 2008). \(\text{NO}_3^-\) represents quantitatively a minor N compound in dry seeds and its accumulation is due to a high-affinity \(\text{NO}_3^-\) transporter, NRT2.7, specifically expressed in seeds (Chopin et al., 2007). One-hour-imbibed seeds of transformants expressing a fusion between NRT2.7 promoter and \(\beta\)-glucuronidase (GUS) reporter gene have shown a GUS staining in the embryo and in the endosperm. Transgenic lines carrying the GFP reporter gene fused to NRT2.7 under the control of the 35S CaMV promoter have evidenced the tonoplastic localization of NRT2.7. \(\text{NO}_3^-\) is not only an important N nutrient for plants but also a higher dormancy (Alboresi et al., 2005). Mutants deficient in NRT2.7 display lower \(\text{NO}_3^-\) content in dry seeds but also a higher dormancy highlighting the signalling role of \(\text{NO}_3^-\) in dormancy relief (Chopin et al., 2007).

Secondary metabolites such as flavonoids are synthesized during seed development and are accumulated in the seed coat and in the embryo (Lepiniec et al., 2006). Flavonoids are polyphenolic compounds responsible for the brown seed colour. They have also important functions in various aspects of seed development and have health benefits when present in animal and human diet (Lepiniec et al., 2006). Flavonoids are involved in protection of seeds against biotic and abiotic stresses, for instance against ultraviolet radiations, and in acting as scavengers of free radicals. The physiological functions of flavonoids in strengthening seed dormancy and viability have also been documented (Debeaujon et al., 2000). Proanthocyanidin (PA) oxidation generates quinones that behave as toxic compounds against pathogens. They also constitute an antinutritive barrier against herbivores and interfere with fungal enzymes necessary for plant cell invasion. Quinones can also act as antioxidants by scavenging reactive oxygen species (ROS) produced by UV radiation, for example (reviewed in Pourcel et al., 2007). Arabidopsis seeds contain flavonols (glycosylated aglycones derivatives) in the seed coat and embryo, and PAs or condensed tannins in the inner integument and chalaza zone (Pourcel et al., 2005; Routaboul et al., 2006). The biosynthesis pathway and regulations have been largely studied especially through transparent testa (tt) mutants (Lepiniec et al., 2006), which are characterized by a lighter seed colour phenotype. The brown colour of Arabidopsis seeds occurring during desiccation is due to the oxidation of PAs and their epicatechin monomers by the laccase-like enzyme TT10/LAC15 (Pourcel et al., 2005). Moreover oxidized PAs cross-link with cell-wall components, thus becoming insoluble and as such difficult to extract (Pourcel et al., 2007). Seeds from the tt10 mutant deprived of TT10 laccase-like activity are yellow at harvest but slowly darken with storage time through chemical oxidation reactions. They exhibit more soluble (i.e. extractable) PAs than wild-type seeds but are not affected in PA biosynthesis per se. They also accumulate less biflavonols, which are dimers of the flavonol quercetin 3-O-rhamnoside and are also synthesized by TT10. Before oxidation, PA biosynthesis and polymerization involve transport and/or vesicle trafficking (Zhao et al., 2010). While the biosynthesis of PA precursors is believed to occur in the endoplasmic reticulum, transfer into the vacuole is performed by TT12 (a multidrug and toxic efflux transporter family) coupled to AHA10 a putative P-type H+ ATPase (Baxter et al., 2005; Marinova et al., 2007). However, the complete story of PA transport inside the cell has not yet been completely elucidated (reviewed in Zhao et al., 2010).

This work describes a new phenotype for the nrt2.7-2 mutant allele which exhibited seeds with more soluble PAs. Little is known about the mechanisms regulating the oxidation of tannins in seeds, and this study provides a new link between nitrogen signalling and PA metabolism. The role of \(\text{NO}_3^-\) accumulated in seeds is discussed in relation to tannin oxidation, TT10 expression, and TT10 activity.

**Materials and methods**

**Plant material**

The nrt2.7-2 homozygous mutant line (EK19) previously isolated from a T-DNA-mutagenized population of Arabidopsis Wassilewskija (Ws) accession in the Versailles transformant library, and the homozygous nrt2.7.1 (SALK_07338) in Columbia (Col) background obtained from the ABRC stock centre (http://signal.salk.edu/cgi-bin/tdnaexpress), were both described in Chopin et al. (2007). The complemented lines nrt2.7-2 C12-3 and nrt2.7-2 C14-6 were obtained after transformation of the nrt2.7-2 mutant by a full-length AtNRT2.7 cDNA placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter according to the method described in Chopin et al. (2007). The tt10-2 mutant (CPI13 line of the Ws ecotype) was described in Pourcel et al. (2005) and the tt4-8 mutant in Debeaujon et al. (2003). The nrt2.7-2 tt10-2 double mutant was generated by crossing the single T-DNA-inserted mutants nrt2.7-2 and tt10-2. F1 plants were grown and self-fertilized to produce a population of F2 plants and the double null mutants for NRT2.7 and TT10 were determined by PCR using primers as described in Chopin et al. (2007) and Pourcel et al. (2005). The clea-1 and clea-2 are T-DNA mutagenized lines isolated from the Versailles transformant library (Ws ecotype) and have been already described in Monachello et al. (2009).

**Growth conditions**

Plants were grown in a growth chamber at 60% relative humidity with a 16/8 light/dark cycle at 21/17 °C and light intensity 150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). Seeds were sown on sand in 5 × 5 cm pots and plants were
subirrigated three times a week with a complete nutrient solution (10 mM NO\textsubscript{3} \textsuperscript{-}, 5 mM KNO\textsubscript{3}, 2.5 mM Ca(NO\textsubscript{3})\textsubscript{2}, 0.25 mM MgSO\textsubscript{4}, 0.25 mM KH\textsubscript{2}PO\textsubscript{4}, 0.42 mM NaCl, 0.1 mM FeNa-EDTA, 30 \mu M H\textsubscript{2}BO\textsubscript{3}, 5 \mu M MnSO\textsubscript{4}, 1 \mu M ZnSO\textsubscript{4}, 1 \mu M CuSO\textsubscript{4}, and 0.1 \mu M (NH\textsubscript{4})\textsubscript{2}MoO\textsubscript{4}. For the experiments on dry seeds, plants were harvested at the end of the culture, whereas for the seed development experiments, flowers at the beginning of anthesis were tagged every 3 d after fertilization (DAF) on one stalk per plant and then 6–21-d-old siliques were harvested.

For the experiment with varying nitrogen nutrition, plants were subirrigated with 100 mM NO\textsubscript{3} \textsuperscript{-} from the sowing to the flowering stage and then with 0.2, 2, or 100 mM NO\textsubscript{3} \textsuperscript{-}. In the 2 mM nutrient solution until harvest, KNO\textsubscript{3} and Ca(NO\textsubscript{3})\textsubscript{2} concentration was 1.75 mM and 0.125 mM, respectively. In the 0.2 mM nutrient solution, KNO\textsubscript{3} concentration was 0.2 mM, and Ca(NO\textsubscript{3})\textsubscript{2} was replaced with 0.25 mM CaCl\textsubscript{2}.

### Nitrates content measurement

Nitrates content of seeds was determined after extraction in water of 2 mg dry seeds or 1 mg developing seeds excised from siliques and siliques without seeds. The nitrates content was measured spectrophotometrically adjusted from Miranda et al. (2001). The principle of this method is a reduction of nitrates by vanadium (III) combined with detection by the acidic Griess reaction.

C, N, total protein, amino acids, sugar, and fatty acid determination

Total C and N determination were carried out on 1 mg seeds following the Dumas combustion method using a NA 1500 Series 2 CN Fison instrument analyser (Thermoquest) as described in Baud et al. (2001). Free amino acids and sucrose contents were determined after 80% (v/v) ethanolic extraction on batches of 20 seeds according to Baud et al. (2002). Free amino acid content was quantified by the ninhydrin colourimetric analysis according to Rosen (1957). Sucrose was determined enzymatically using a kit (Boehringer Mannheim). Starch was quantified from the pellet resulting of the ethanolic extraction. After hydrolysis of starch by amyloglucosidase and amylase (Baud et al., 2002), glucose was determined enzymatically using a kit (Boehringer Mannheim). Total protein content was determined on batches of 1.5 mg seeds by the ninhydrin colourimetric quantification of the amino acids released after 1 h hydrolysis of the seeds at 120 °C in 3 M NaOH, as described in Baud et al. (2007).

### Flavonoid composition analyses

Flavonoids were extracted from 15 mg dry seeds with acetonitrile/water (75:25, v/v), as described by Routaboul et al. (2006). After centrifugation of the extract, the supernatant was used for the analysis of flavonoids and soluble PAs, while the pellet contained insoluble PAs. Analyses of soluble and insoluble PAs were further performed after acid-catalysed hydrolysis and absorbance measured at 550 nm using cyanidin as a standard molecule according to Routaboul et al. (2006). Epicatechin monomers and PA polymers were then analysed by LC-MS. Flavonol composition was also analysed by LC-MS using apigenin as an internal standard which was added at the time of extraction (Routaboul et al., 2006).

### RNA extraction and gene expression analysis

Total RNA was extracted from excised seeds or siliques having their seeds removed (the material from three siliques at the same development stage were pooled for each extract) with a RNeasy Plant Mini kit (Qiagen). First-strand cDNAs were synthesized from 1 \mu g RNA using Moloney murine leukaemia virus reverse transcriptase (Thermo Scientific) and oligo(dT)15 primers (Thermo Scientific). The absence of DNA contamination was verified by PCR using specific primers spanning an intron in Nii (At2g15620): forward 5′-TGCAGATGACCTTCTCCTGC-3′; reverse 5′-CTGAGGTTGACTCCGAAATA GTTCTC-3′. Gene expression analyses were determined by quantitative real-time PCR (qPCR, Eppendorf Realplex MasterCycler with a Roche LightCycler-FastStart DNA Master SYBR Green I kit, according to the manufacturer’s protocol) using 2.5 \mu l of a 1:5 dilution of first-strand cDNA in a total volume of 10 \mu l. The gene-specific primers were: TT10 (At5g451000): forward 5′-GCAAGATCTTACAAACAAGCGG-3′; reverse 5′-CCCATGACCTCCGATTCC-3′; NRT2.7 (At5g14570): forward 5′-TCTGATCATTTCGCTGGTATT-3′; reverse 5′-AATTCGGTCATGGTGGAGTA-3′; CLca (At5g40890) forward 5′-ATCACATCGAAGTTTAGATT-3′; reverse 5′-AATGTAGTAGCCGACGCAGAA-3′. The results were standardized using two reference genes chosen as the most stable and accurate ones during seed maturation: EF1\textalpha (At5g60390): forward 5′-TGGGAGGTTTGGAGCGGTA-3′; reverse 5′-CCACGATATGCGATGGATGGAAGAATACG-3′; and APC2 (At2g04660: forward 5′-AAAGGATCAGCCACA CAAAACATC TTG-3′). The expression of each gene was normalized to the level of a synthetic reference gene (SRG) as follows (Vandesompele et al., 2002)

\[
\%SRG = \frac{\text{Ct}(\text{SRG})}{\text{Ct}(\text{SRG}) \times 10^{(0.7 \times \text{Ct}(\text{SRG}) - 16.25) - 1}}
\]

### In situ TT10 activity

The in situ enzymatic activity of TT10 was measured as described in Pourcel et al. (2005). The accelerated browning assay was performed on immature seeds (7–8 DAF) in 100 mM phosphate buffer pH 6.6, 50 mM epicatechin (Sigma-Aldrich). Vacuum was applied for 1 h before incubation at 37 °C in the dark overnight. Seeds were observed directly under a binocular for relative browning intensities.

### Results and discussion

Soluble PAs are more accumulated in seeds of nrt2.7-2 mutant

Because NO\textsubscript{3} \textsuperscript{-} is an important N nutrient for plants, the impact of the lack of NRT2.7 on the accumulation of other N and C reserve compounds was evaluated in nrt2.7 mutant seeds when grown on nonlimited supply of N (10 mM NO\textsubscript{3} \textsuperscript{-}). Total N and free amino acid contents were not affected in the nrt2.7-2 mutant compared to the wild type (Table 1), while total protein content was slightly increased (Table 1) and NO\textsubscript{3} \textsuperscript{-} content was decreased (Fig. 1A). A decrease in NO\textsubscript{3} \textsuperscript{-} content was also observed in tt10-2 mutant seeds (Fig. 1A). In contrast, total C, fatty acids, starch, and sugar contents were not changed in the nrt2.7-2 mutant (Table 1). Thus, the decrease in capacity to store NO\textsubscript{3} \textsuperscript{-} in nrt2.7-2 seed vacuole seemed to favour the accumulation of N-protein reserve compounds without affecting C content.

Interestingly the nrt2.7-2 mutant seeds displayed a slightly lighter colour compared to the wild-type Ws, resembling the phenotype of tt10 mutant seeds (Pourcel et al., 2005) (Fig. 2). This lighter phenotype was also observed in the
double mutant nrt2.7-2 tt10-2 with a pale-brown seed coat colour and a dark-brown chalaza zone (Fig. 2). The analysis of flavonoids in mature seeds revealed that the soluble PA content was similarly increased in the nrt2.7-2 and tt10-2 mutants compared to Ws seeds (Fig. 1A) while insoluble PAs were not changed (Supplementary Fig. 1, available at JXB online). LC-MS analyses showed that the soluble epicatechin monomers and oligomers were also increased in the nrt2.7-2 mutant (Fig. 1B) as well as in the tt10-2 mutant (see Pourcel et al., 2005). However, unlike the tt10-2 mutant that contains only very small amounts of biflavonols (dimer of quercetin 3-O-rhamnoside) and a slightly more quercetin 3-O-rhamnoside monomers (Fig. 1C), the flavonol composition of the nrt2.7-2 mutant was not modified (Fig. 1C). Thus, the nrt2.7-2 mutant was very peculiar as it exhibited a modification in flavonoid composition that was specific for PAs and perhaps, as in the tt10 mutant, linked to a defect in PA oxidation. It remains to be investigated whether the TT10 function is altered in nrt2.7-2 or whether another oxidative mechanism is involved.

To first confirm the link between the T-DNA insertion in At5g14570 and the phenotype of nrt2.7-2, a functional complementation of nrt2.7-2 was conducted with a construct, Pro35S:AtNRT2.7, which allows overexpression of a full-length NRT2.7 under control of the CaMV 35S promoter. As a result, the soluble PAs and nitrate content phenotypes

Table 1. Analysis of mature seeds from Ws and the nrt2.7-2 mutant

| Parameter                  | Ws         | nrt2.7-2   |
|----------------------------|------------|------------|
| N (% seed dry weight)      | 3.75 ± 0.04| 3.78 ± 0.01|
| Total protein (μg BSA (mg seed)⁻¹) | 140.53 ± 6.63 | 181.98 ± 13.04* |
| Soluble amino acids (nmol (mg seed)⁻¹) | 10.68 ± 3.25 | 11.01 ± 4.41 |
| C (% seed dry weight)      | 54.04 ± 0.31| 53.19 ± 0.24|
| Fatty acids (μg (mg seed)⁻¹) | 378.27 ± 1.46 | 371.44 ± 4.15 |
| Sucrose (nmol (mg seed)⁻¹) | 35.88 ± 1.94 | 34.59 ± 0.40 |
| Starch (eq nmol Glu (mg seed)⁻¹) | 2.60 ± 0.17 | 2.48 ± 0.22 |

* indicates significant differences between the wild type (Wassilewskija, Ws) and the nrt2.7-2 mutant (Student t-test P<0.05).

![Fig. 1. Nitrate content and flavonoid composition of tt10-2, nrt2.7-2, nrt2.7-2 C12, nrt2.7-2 C14, and wild-type (Ws) mature seeds. (A) Analysis of soluble proantocyanidins (PAs) after acid-catalysed hydrolysis and determination of nitrate content. (B) Analysis of epicatechin (EC) monomers and oligomers (B2 and trimer) by LC-MS. (C) Analysis of flavonol composition by LC-MS. G, Glucoside; H, hexoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside. Values are mean±standard error of seeds of three individual plants. Statistical analysis was performed using analysis of variance and the means were classified using Tukey HSD test (P<0.05): (A) a, b, c, different letters above bars indicate statistically significant differences; (B, C) *** indicates significant differences between Ws and the nrt2.7-2 mutant or between Ws and the tt10.2 mutant (Student t-test P<0.001).
were both restored in the two complemented lines 
rt2.7-2 C12 and 
rt2.7-2 C14 (Fig. 1A). However, the PA phenotype of the 
rt2.7-2 mutant allele was specific for the Ws accession, since no difference in soluble PAs was observed in the 
rt2.7-1 null mutant allele in Columbia background (Supplementary Fig. S2 available at JXB online) whereas the nitrate phenotype was also encountered in 
rt2.7-1 (Chopin et al., 2007). These data suggest that nitrate and soluble PA contents are not directly correlated. The specificity of the PA phenotype for the Ws accession was surprising but natural variability in PA accumulation has already been reported (Lepiniec et al., 2006; Routaboul et al., 2012), suggesting a variability in the regulation of PA oxidation. Besides, plant nitrate content varies also among accessions and, more precisely, Col accession displays a higher capacity to store nitrate than Ws accession in seeds (Chopin et al., 2007) and in foliar tissues (North et al., 2009), and consequently Col is more tolerant to N limitation. Control of PA oxidation originating from natural diversity of strategies for nitrate use and storage might explain the lack of PA phenotype for the Col accession.

Thus, this work investigated further the relationship between nitrate accumulated in the seed and condensed PA accumulation. Since the PA phenotype of the 
rt2.7-2 mutant was first observed when plants were grown on nonlimiting supply of N nutrition (10 mM) as described above, a more comprehensive range of NO$_3^-$ nutrition was also tested from 0.2 and 2 mM NO$_3^-$ as limited N levels to 10 mM NO$_3^-$, The NO$_3^-$ content of dry seeds was linked to the NO$_3^-$ nutrition in both genotypes and it was lower in the 
rt2.7-2 mutant than in Ws on 10 mM NO$_3^-$, but not significantly affected on 2 and 0.2 mM NO$_3^-$ (Fig. 3A). Epicatechin and soluble PAs (epicatechin oligomers) were more accumulated in the 
rt2.7-2 mutant for all nutrition levels (Fig. 3B and C), while still no change was observed for flavonols (Fig. 3D). The effect of 
rt2.7-2 mutation on both NO$_3^-$ and soluble PA contents increased with the NO$_3^-$ nutrition level. Considering these results, subsequent experiments were performed at 10 mM NO$_3^-$, which allowed viewing of the most pronounced flavonoid and nitrate phenotypes.

The NO$_3^-$ content in seeds was dependent on supply of NO$_3^-$ nutrition (Fig. 3A) and thus may be relevant to the NO$_3^-$ availability for allocation to the seeds. This work speculated whether a limited capacity of NO$_3^-$ storage in leaves could also modulate NO$_3^-$ transfer to the seeds and could also influence the soluble PA level in seeds. Therefore, this work analysed the consequence of a knockout mutation in CLCa, encoding a nitrate/proton antiporter responsible for NO$_3^-$ accumulation in vacuolar compartment in leaves (Monachello et al., 2009). Interestingly, NO$_3^-$ content was decreased in clca1 and clca2 mutant seeds to the same extent as in the 
rt2.7-2 mutants, while the soluble PA accumulation was not changed in clca1 and clca2 mutants (Ws background) (Fig. 4A and B). This result suggested that the mechanism linking NO$_3^-$ accumulation and PA accumulation in seeds was specifically linked to 
RT2.7 function in seeds rather than to global NO$_3^-$ accumulation.

Nitrate accumulation during seed development

It has already been described that PA oxidation in the testa starts with the desiccation of developing seeds (Pourcel et al., 2005). In order to better understand the link between 
RT2.7 and PA oxidation/accumulation in seeds, the current work investigated more precisely the fluctuation of NO$_3^-$ content in seeds and in siliques tissues (siliques excluding seeds) during seed development. The NO$_3^-$ content was the highest in young seeds (9 DAF) and decreased abruptly (12 DAF) to the final low content in mature seeds (Fig. 5A). Conversely NO$_3^-$ content was the lowest in young siliques tissues (9 DAF) and increased regularly up to the senescing stage (21 DAF) (Fig. 5B). In the 
rt2.7-2 mutant, the NO$_3^-$ contents were slightly lowered in seeds at 12 DAF and in mature seeds compared to those in Ws (Fig. 5A), concomitantly to the maxima of 
RT2.7 expression in Ws (Fig. 5C). In contrast, NO$_3^-$ content was not affected in siliques tissues of the 
rt2.7-2 mutant (Fig. 5B). Thus, 
RT2.7 was likely not the only actor responsible for NO$_3^-$ accumulation in these tissues. According to Almagro et al. (2008), the impact of the 
RT1.6 (AtNPF2.12) mutation was strongly associated with a reduced NO$_3^-$ content in seeds and an increased seed abortion, but no colour phenotype of the 
rt1.6 mutant seeds was reported. In the current study, no significant difference in 
RT1.6 (AtNPF2.12) expression was measured in Ws and in 
rt2.7-2 (data not shown). 
RT1.6 (AtNPF2.12) was expressed in the vascular tissue of the siliques and funiculus and was partially responsible for the delivery of NO$_3^-$ into the seed, but 
RT1.6 (AtNPF2.12) was localized at the plasma membrane and, thus, may not be able to compensate the vascular nitrate storage in 
rt2.7-2. Expression of the vascular anionic
channel CLCa was detected in siliques (Fig. 5D) and, thus, could explain the partial compensation mechanism for the loss of NRT2.7 function in this organ, but no expression of CLCa was measured in excised seeds (Fig. 5C). Further study is required to find out if any other transporter is functional in these organs.

The PA phenotype of the nrt2.7-2 mutant is not due to a modulation of T710 expression

nrt2.7-2 mutant seeds accumulated less NO$_3^-$ and more soluble PAs and epicatechins compared to Ws partially resembling tt10 mutant phenotype. Thus, this phenotype was likely arising from a defect in PA oxidation leading to an accumulation of soluble forms of PAs during the development. According to Pourcel et al. (2005), T710 expression in entire siliques begins to be detected at 4 DAF. Thus, the current work investigated T710 and AtNRT2.7 expression in excised seeds and siliques excluding seeds of Ws and the nrt2.7-2 mutant during seed development. In Ws, the level of NRT2.7 mRNA was lower than T710 but they were expressed in seeds and siliques (Fig. 5C and 5D). The expression patterns of T710 and NRT2.7 varied along seed development. T710 expression was repressed in excised seeds from 9 DAF to 21 DAF (or mature seeds) (Fig. 5C). T710 mRNA levels in siliques were measured 50% lower than those in excised seeds (Fig. 5D). In contrast, NRT2.7 expression showed two maxima in excised seeds, at 12 and 21 DAF (Fig. 5C) and increased slightly in siliques from 9 to 18 DAF (Fig. 5D). Furthermore, this work failed to observe a modified expression pattern of T710 that was significantly reproducible in the nrt2.7-2 mutant compared to Ws (data not shown).

A role in signalling was previously suggested for NO$_3^-$ in relieving seed dormancy (Alboresi et al., 2005). However, considering that the maximum of T710 expression preceded the first raise in NRT2.7 expression and the beginning of NO$_3^-$ content to decrease in nrt2.7-2, the current study excluded the hypothesis of a signalling role for NO$_3^-$ in downregulating the expression of T710 and then lowering soluble PA oxidation.
Is the PA phenotype of the nrt2.7-2 mutant due to a modulation of TT10 activity?

In order to find out a causal explanation for the PA phenotype of the nrt2.7-2 mutant, TT10 activity was considered. The enzymic activity of TT10 has never been successfully measured in vitro but an assay for in situ detection of browning in immature seed coat has been reported by Pourcel et al. (2005). In a first attempt, the current study looked into the in situ measurement of TT10 activity in young seeds (7–8 DAF) ofWs and the nrt2.7-2 mutant using the tt10-2 mutant as a negative control and the tt4-8 mutant as a positive control without endogenous supply of flavonoids (due to the lack of chalcone synthase). The browning intensity of the seeds incubated in presence of the epicatechin substrate revealed the PA oxidation activity of TT10. As expected, tt10-2 seeds stayed colourless and seeds of Ws and tt4-8 showed a brown colour, but nrt2.7-2 seeds became as brown as Ws (Table 2). These results suggested that the oxidative activity of TT10 was not altered in nrt2.7-2 seeds at this stage. However, this type of experiment is only feasible when the testa was still colourless in immature seeds. At this stage TT10 was highly expressed but these conditions were not favourable for a maximal NRT2-7 expression. Further investigation of TT10 activity by optimizing the in situ measurement at older stages is needed to understand the mechanism of higher soluble PA accumulation in nrt2.7-2 seeds.

Since the mechanisms for regulating the TT10 activity are largely unknown, the link between NRT2.7 and TT10 activity is difficult to assess. TT10 protein has been described as a putative laccase containing four His-rich copper-binding domains, corresponding to the putative catalytic sites of the multi-copper oxidase family (Pourcel et al., 2005). A phylogenetic analysis has revealed the highest homology of TT10 with four other dicotyledonous laccases (and for example with RvLAC2 from the sap of the Japanese lacquer tree Rhus vernicifera). Nitric oxide (NO) has been reported as a regulator of laccases, acting as a reducer of the R. vernicifera laccase RvLAC2 and also of fungal laccases (Torres and Wilson, 1999; Wilson and Torres, 2004). However, the consequences of the NO action on the enzymic activity of laccase are not completely understood (Torres et al., 2002). TT10 protein has recently been experimentally shown to be localized in the vacuole (Pang et al., 2013), the same cellular compartment as NRT2.7, but a hypothetical link between NO, TT10 activity, and NRT2.7 remains uncertain.

What is a role for NRT2.7 in PA oxidation/accumulation?

According to Pourcel et al. (2005), TT10 is expressed in the developing testa, firstly in the inner integument (PA-producing cells) and afterwards in the outer integument (location of flavonol synthesis). NRT2.7 expression has been previously localized in the endosperm and in embryo in imbibed seeds (Chopin et al., 2007) while PAs are synthesized and accumulated in the endothelium. Although the current work was able to measure NRT2.7 expression by qPCR in excised seeds, all attempts viewing the localization of NRT2.7 in the seed during its development by in situ hybridization or immunolocalization were unsuccessful. However, NRT2.7 expression is present in the seed coat according to the data available on the eFP browser web site (http://wwwiggins.utoronto.ca/efp_seedcoat/cgi-bin/efpWeb.cgi). NRT2.7 has already been described as a NO3- transporter (Chopin et al., 2007), which is coherent with the lower NO3- content in seeds of nrt2.7 mutant. Since the PA phenotype appeared more strictly correlated to the presence of NRT2.7 than to the vacuolar NO3- content (Fig 3A and B), it was speculated whether the function of NRT2.7 in PA oxidation could be related to another function of NRT2.7 hitherto unknown. It has been demonstrated that NRT1 (NPF) proteins are able to transport molecules other than nitrate (Lérant et al., 2013), although little is known about the NRT2 family. Further experiments are needed in order to ascertain such hypothesis. The transport of epicatechin into and out of the vacuolar compartment could have been disturbed in absence of NRT2.7. TT12 is a MATE transporter involved in the storage of PA precursor into the vacuole and its activity is coupled to AHA10, an H+-ATPase. Aha10 and tt12 mutants are affected...
in PA accumulation and also in the vacuolar biogenesis, supporting an endomembrane function for these transporters. There may be a direct or indirect link between these transport activities and NRT2.7 that involves pH stability, tonoplast stabilization, or other unknown mechanism.

**Supplementary material**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Analysis of insoluble PAs of *nrt2.7-2* and wild-type mature seeds after acid-catalysed hydrolysis.

**Table 2.** In situ enzymic activity of the TT10 laccase in the wild type and mutants

The analysis was performed according to the method described in Pourcel et al. (2005). The table describes seed coat colour with and without (control) the addition of epicatechin substrate to immature seeds (7–8 DAF). The browning colour intensity is positively correlated to TT10 activity. It is recorded by visual observation and noted as such: –, colourless; +++<++++, increasing browning colour. Approximately 50 seeds per sample were analysed.

| Substrate | Ws | nrt2.7-2 | tt10.2 | tt4.8 |
|-----------|----|---------|-------|------|
| Control   | –  | –       | –     | –    |
| Epicatechin| +++| +++     | –     | +++  |

**Fig. 5.** Nitrate content and gene expression in developing seeds from 9 to 21 d after flowering (DAF). (A, B) Nitrate content of *nrt2.7-2* and wild type in excised seeds (Ws) (A) and in siliques emptied from their seeds (silique tissues) (B). (C, D) Expression of TT10, NRT2.7, and CLCa of Ws in excised seeds (A) and silique tissues (B). Each gene expression data was normalized to the level of a synthetic reference gene (SRG) using reference genes EF1a and APC, as described in Materials and methods. Values are mean±standard error of seeds of three individual plants. NA, not analysed.

**Supplementary Fig. S2.** Analysis of soluble PAs of *nrt2.7-1* and wild-type mature seeds (Col accession).

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