Involvement of the Putative N-Acetylornithine Deacetylase from Arabidopsis thaliana in Flowering and Fruit Development

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In eukaryotic cells, the non-proteinogenic amino acid ornithine is the precursor of arginine and polyamines (PAs). The final step of ornithine biosynthesis occurs in plants via a cyclic pathway catalyzed by N²-acetylornithine:N-acetylglutamate acetyltransferase (NAOGAcT). An alternative route for ornithine formation, the linear pathway, has been reported for enteric bacteria and a few other organisms; the acetyl group of N²-acetylornithine is released as acetate by N²-acetylornithine deacetylase (NAOD). NAOD activity has never been demonstrated in plants, although many putative NAOD-like genes have been identified. In this investigation, we examined the effect of down-regulation of the putative Arabidopsis thaliana NAOD gene by using AtNAOD-silenced (sil#17) and T-DNA insertion mutant (ataaod) plants. The ornithine content was consistently reduced in sil#17 and ataaod plants compared with wild-type plants, suggesting that in addition to NAOGAcT action, AtNAOD contributes to the regulation of ornithine levels in plant cells. Ornithine depletion was associated with altered levels of putrescine and spermine. Reduced AtNAOD expression resulted in alterations at the reproductive level, causing early flowering and impaired fruit setting. In this regard, the highest level of AtNAOD expression was observed in unfertilized ovules. Our findings suggest that AtNAOD acts as a positive regulator of fruit setting and agree with those obtained in tomato auxin-synthesizing parthenocarpic plants, where induction of SINAOD was associated with the onset of ovary growth. Thus, here we have uncovered the first hints of the functions of AtNAOD by connecting its role in flower and fruit development with the regulation of ornithine and PA levels.

Keywords: Early flowering • Flower fertility • Fruit development • Ornithine • Polyamines.

Abbreviations: ADC, arginine decarboxylase; GA₃, gibberellic acid; GUS, β-glucuronidase; MS, Murashige and Skoog; NAOD, N²-acetyl ornithine deacetylase; NAOGAcT, N²-acetylornithine:N-acetylglutamate acetyltransferase; ODC, ornithine decarboxylase; PA, polyamine; Put, putrescine; qRT–PCR, quantitative reverse transcription–PCR; Spd, spermidine; Spm, spermine.

Introduction

The amino acids ornithine and arginine are nitrogen storage compounds, serving as precursors of the polyamines (PAs) that are implicated in a wide range of growth and developmental events in most organisms. Ornithine itself is also an intermediate for the biosynthesis of arginine (Slocum 2005).

The simplest PA, putrescine (Put), can be formed in plants either directly from ornithine in a reaction catalyzed by ornithine decarboxylase (ODC) or indirectly from arginine by arginine decarboxylase (ADC) via agmatine (Walden et al. 1997). An exception is represented by Arabidopsis thaliana, where Put is apparently produced only by ADC, since ODC activity has not been detected in this species (Hanfrey et al. 2001). Put can then be converted to spermidine (Spd) and spermine (Spm) by the sequential activities of spermidine synthase and spermine synthase, with the use of decarboxylated S-adenosylmethionine as the aminopropyl donor. In plants, PAs play a crucial role in cellular and developmental processes, including the control of cell division, flowering, fruit development, root formation and senescence (Carbonell and Navarro 1989, Evans and Malmberg 1989, Imai et al. 2004, Liu et al. 2006, Tiburcio et al. 2014).

Two alternative routes have been proposed for ornithine biosynthesis. In the cyclic pathway, ornithine is formed via the activity of N²-acetylornithine:N-acetylglutamate acetyltransferase (NAOGAcT), which recycles the acetyl group of N²-acetylornithine on glutamate, regenerating N-acetylglutamate. This pathway is peculiar to plants, non-enteric bacteria and fungi (Shargool et al. 1988, Slocum 2005).

In the linear pathway, ornithine synthesis is catalyzed by N²-acetylornithine deacetylase (NAOD) (Cunin et al. 1986, Shargool et al. 1988, Caldovich and Tuchman 2003, Slocum 2005). The acetyl group of N²-acetylornithine is not recycled to glutamate, but is released as acetate. The linear pathway has been detected in enteric bacteria (Meinnel et al. 1992), and in a...
few other organisms such as thermophilic bacteria (Marc et al. 2000) and yeast (Crabeel et al. 1997).

The NAOD from *Escherichia coli*, encoded by ArgE, is a homodimer of monomers with a subunit molecular mass of 42 kDa (Meinnel et al. 1992, McGregor et al. 2005). ArgE is a metallopeptidase that is activated by cobalt and inorganic phosphate (Sakanyan et al. 1993, Javid-Majd and Blanchard 2000).

ArgE belongs to a large family of enzymes that catalyze the hydrolysis of the peptidic or N-acetyl bond to yield free amino acids as products. This family also includes DapE (diaminopimelic acid deacetylase) from *E. coli*, Cpg2 (carboxypeptidase G2) from *Pseudomonas* sp., the porcine Acy1 (aminocyclase) and YscS (carboxypeptidase S) from *Saccharomyces cerevisiae* (Boyen et al. 1992, Meinnel et al. 1992, Biagini and Puigserver 2001, Slocum 2005).

NAOD activity has not been demonstrated in plants, which are consequently considered unable to use the linear pathway for ornithine formation (Slocum 2005, Page et al. 2012, Frémont et al. 2013). However, many putative NAOD-coding sequences have been identified in plants. Some of these proteins exhibit modulation in response to environmental and developmental conditions. High levels of ArgE homologs were measured in wild watermelon leaves in response to drought stress (Kawasaki et al. 2000). An aminoacylase similar to NAOD was found in ripened papaya fruits compared with unripe fruits (Fabi et al. 2012). A putative tomato NAOD was identified in a transcriptomic analysis comparing parthenocarpic and wild-type flower buds collected at pre-anthesis (Molesini et al. 2009a). The parthenocarpic tomato plants used in that analysis were engineered by introducing auxin-synthesizing genes under the control of an ovary-specific promoter (Ficcadenti et al. 1999, Pandolfini et al. 2002). Since successful fertilization of the ovule leads to a localized burst of auxin that depresses ovary growth, the expression of auxin-synthesizing genes in transgenic flower buds induces early fruit setting independently of fertilization (Rotino et al. 1997, Ficcadenti et al. 1999, Pandolfini et al. 2002). Among the 266 CDNA clones differentially expressed in tomato parthenocarpic flower buds (Molesini et al. 2009a), a transcript (XM_004245844) encoding a putative NAOD (SINAOD; XP_004245892) appeared to be induced in auxin-synthesizing flower buds compared with wild-type buds. Analysis of SINAOD expression at various stages of wild-type flower/fruit development revealed its strong accumulation at pre-anthesis and its drastic decrease after fertilization (Molesini et al. 2009a, Pandolfini et al. 2009). The expression pattern of SINAOD in parthenocarpic and wild-type plants might be indicative of a role for this gene in fruit initiation (Molesini et al. 2009a, Pandolfini et al. 2009).

In the present investigation, we studied the role of At4g17830, a gene encoding a putative Arabidopsis NAOD. Through the use of a loss-of-function T-DNA insertion mutant and a silenced line, we determined that AtNAOD participates in the regulation of flowering time and fruit setting. Ornithine content was consistently lower in the mutant and silenced plants than in wild-type plants. In addition, the levels of Put and Sprm were significantly altered. Our results add new insights into the role of PAs and nitrogen storage compounds in reproductive metabolism.

## Results

**AtNAOD encodes a putative acetylornithine deacetylase highly expressed in pistils**

The Arabidopsis genome contains a single gene (At4g17830), located on chromosome IV and organized into nine exons, which encodes two putative NAODs of 440 (Q9CS5C4) and 445 (F4JPZ7) amino acids (Fig. 1). The shorter protein (hereafter annotated as AtNAOD), which is 99% identical to the longer one, contains the same exon combination as the longer protein but differs in the length of the second exon (Fig. 1). BLASTP (Altschul et al. 1997) analysis revealed that AtNAOD displays a 73% and 69% degree of identity with sequences identified in tomato (XP_004245892; Molesini et al. 2009a) and watermelon (Q9MB49; Kawasaki et al. 2000), respectively (Fig. 1). The phylogenetic relationships revealed that AtNAOD is most closely associated with other Brassicaceae species (Supplementary Fig. S1). AtNAOD shares 25% of the amino acid sequence of *E. coli* ArgE (P23908) (data not shown). Further analysis of the AtNAOD protein primary structure (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al. 2005) predicted a domain characteristic of the M20 peptidase family (amino acids 15–437), with similarity to known NAODs. No signal peptides for organellar targeting were predicted (Target P; Emanuelsson et al. 2000).

AtNAOD expression in various adult organs (bolts, leaves, inflorescences, roots and green siliques) was monitored via quantitative reverse transcription–PCR (qRT–PCR). AtNAOD was expressed at a low level in all vegetative organs; the highest expression was detected in green siliques (Fig. 2A). To visualize the tissue- and development-specific expression patterns of AtNAOD in more detail, we constructed a β-glucuronidase (GUS) reporter by fusing a 1,053 bp fragment upstream of the AtNAOD translatable start codon (AtNAODp::GUS; Supplementary Fig. S2A) to the coding region of *E. coli* uidA. An in silico analysis of the chosen promoter region in the PlantPAN database (Chang et al. 2008) revealed the presence of several putative binding sites for transcription factors implicated in the identity of the floral meristem (Supplementary Fig. S2B). AtNAODp::GUS plants were histochemically stained with 5-bromo-4-chloro-3-indolyl-β-glucurononic acid at various flower/fruit developmental stages as well as in vegetative organs (Fig. 2B). No detectable GUS activity was observed in vegetative organs under our experimental conditions (data not shown). GUS activity and thus AtNAOD expression during flower and fruit development was detectable only in a few stages of pistil development (Fig. 2B). AtNAOD expression was mainly localized to unfertilized ovules present in flowers after anthesis and in green siliques. GUS staining was not visible throughout the ovule, but appeared to be restricted to the embryo sac. Stamens did not display visible GUS staining (data not shown). Thus, this pattern of expression suggests that AtNAOD is associated with ovule development.
Phenotypic characterization of the T-DNA mutant and the AtNAOD-silenced line revealed early flowering and impaired fruit setting

In parthenocarpic tomatoes, we previously observed that SlNAOD expression was precociously up-regulated in flower buds, concomitant with fruit initiation (Pandolfini et al. 2009). Also taking into consideration the remarkable expression of AtNAOD in ovules, we hypothesized that NAOD may positively regulate fruit setting, and that knocking out this gene would result in alterations at the reproductive level.

Functional analysis of AtNAOD was carried out via T-DNA insertion and AtNAOD silencing. Two putative knock-out T-DNA insertional lines in At4g17830 were obtained, SALK_108570 from the SALK collection (Alonso et al. 2003) and...

Fig. 1 Sequence alignment of putative plant NAODs. The amino acid sequences of putative NAODs from Arabidopsis (F4JPZ7 and Q9C5C4), watermelon (Q9MB49) and tomato (XP_004245892.1) were aligned using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) program, choosing default alignment parameters and selecting BLOSUM for the protein matrix. Consensus symbols: '*' identical residues; ':' residues with strongly similar properties; ':' residues with weakly similar properties.

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and 846C09 from the GABI-Kat collection (Kleinboelting et al. 2012). Two homozygous lines were selected after PCR. The mutant line SALK_108570 has a T-DNA insertion in the AtNAOD promoter, 434 nucleotides upstream of the 5’-untranslated leader region; in this line, the AtNAOD mRNA level was unaffected (data not shown). Line GABI-Kat 846C09 (hereafter annotated as atnaod) harbors a T-DNA insertion in the third exon (20 nucleotides downstream of the 5’ end of the exon). Transcript levels of AtNAOD in atnaod plants were found to be 85% lower than those in wild-type plants (Fig. 3A) when evaluated by qRT–PCR with primers downstream of the T-DNA insertion. Down-regulation of AtNAOD expression was also

Fig. 2 Analysis of the AtNAOD expression pattern. (A) Quantitative RT–PCR of AtNAOD in various tissues of wild-type adult plants. The expression levels were normalized using actin as the endogenous control gene and the relative expression ratios were calculated using bolts as the calibrator sample. The values reported are means ± SE (n = 3); Student’s t-test was applied, ***P < 0.001 vs. bolts. (B), Histochemical localization of AtNAODp::GUS expression during Arabidopsis flower and fruit development. (a) Early stage of flower development (around stage 12); (b) flower at anthesis (stage 13) and close-up view of ovules; (c) flower after anthesis, and close-up view of unfertilized ovules showing GUS signal localization in the embryo sac; (d) fertilized and unfertilized ovules present in green growing silique; GUS activity is detected in unfertilized ovules.
obtained by stably transforming Arabidopsis with a hairpin construct to elicit RNA silencing. Among the eight transgenic lines obtained, we selected the two lines, sil#2 and sil#17, that displayed the strongest AtNAOD mRNA suppression (approximately 90% reduced) compared with the level in wild-type plants. The expression levels were normalized using actin as the endogenous control gene. The values are means ± SE (n = 3). Student's t-test was applied. **p < 0.01; ***p < 0.001 vs. the wild type.

We first examined seedling growth of atnaod and AtNAOD-silenced lines; no visible alterations in the germination rate or seedlings development were observed compared with the wild-type line (data not shown).

We evaluated fruit development in the atnaod mutant and in the two silenced lines and observed decreases in the number and length of mature siliques compared with wild-type siliques (data not shown). These results motivated us to examine the reproductive behavior of the atnaod mutant and sil#17 lines more closely. Both the mutant and the silenced lines displayed an early-flowering phenotype compared with the wild-type line (Fig. 4A). Thirty days after sowing, the first flower buds were visible in approximately 74% and 34% of the atnaod mutant and sil#17 plants, respectively, but only in 13% of the wild-type plants (Fig. 4B). Three days later, 93% of atnaod mutant plants, 63% of sil#17 plants and 44% of wild-type plants showed flower buds and/or flowers (Fig. 4B). The average time to reach the stage with the first visible flower buds (Boyes et al. 2001) was 29, 31 and 33 d after sowing for atnaod, sil#17 and wild-type plants, respectively (Fig. 4C).

To assess further the early flowering phenotype of atnaod and sil#17 plants, we recorded the total number of leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time that the first flower opened (Gómez-Mena et al. 2001). At this stage, atnaod mutant plants and sil#17 plants had fewer leaves than wild-type plants (Fig. 4D). Except for the earliness in flowering, the flowers of the atnaod mutant and sil#17 were wild type in appearance (data not shown).

We also evaluated the fruit setting capacity of AtNAOD-down-regulated plants by monitoring the number of aborted siliques (≤0.5 cm) on the main bolt. Both atnaod and sil#17 plants had very high numbers of aborted siliques, 5–6 times higher than wild-type plants (Fig. 5A, B). In terms of the growth and seed production of fertilized siliques, we observed a slight reduction in siliques length in both atnaod and sil#17 plants (~4% and ~13%, respectively) vs. wild-type plants (Fig. 5C).

As expected, the average number of seeds per siliques was reduced by approximately 7% and 12% in the atnaod and sil#17 plants, respectively (Fig. 5D). Our findings imply that AtNAOD may be required for full fruit and seed setting.

Considering that auxins and gibberellins are key determinants of the initial stages of fruit development in Arabidopsis, and that a NAOD-like gene was differentially expressed in auxin-hypersynthesizing flower buds in tomato (Pandolfini et al. 2009, Molesini et al. 2009a, Sotelo-Silveira et al. 2014), we tested the sensitivity of atnaod and sil#17 plants to the naturally occurring IAA and to gibberellic acid (GA3).

To assess whether AtNAOD affects auxin responses, we used a root growth inhibition test to determine the root phenotypes of atnaod and sil#17 plants. The application of low concentrations of exogenous auxin (≤0.1 μM) inhibits primary root growth and generally increases the density of lateral roots in wild-type plants (Ruegger et al. 1998, Casimiro et al. 2001). atnaod and sil#17 seedlings grown for 7 d in medium supplemented with a low concentration of IAA (0.03 and 0.1 μM) showed a normal response both in primary root growth and in lateral root density compared with wild-type seedlings (Supplementary Fig. S3). To test whether atnaod and sil#17 plants were altered in their sensitivity to GA3, we examined hypocotyl elongation, which under light is promoted by increasing concentrations of GA3 (Ephritikhine et al. 1999). atnaod and sil#17 seedlings grown for 7 d in vitro with increasing GA3 concentrations (1, 10 and 100 μM) displayed hypocotyl responses (Supplementary Fig. S3) that were similar to the wild-type response. These data indicate that the loss of AtNAOD function does not alter sensitivity to auxin and gibberellin.

Ornithine content was altered in the T-DNA mutant and in the AtNAOD-silenced line

Ornithine, the precursor of arginine, can be produced by a cyclic pathway controlled by the activity of NAOGAcT and
by a linear pathway involving NAOD (Fig. 6A). In the majority of plants, ornithine and arginine can be converted into Put by ODC and ADC, respectively. Arabidopsis apparently lacks the gene encoding ODC (Hanfrey et al. 2001) (Fig. 6A). Hence, in Arabidopsis, ADC is probably the sole route for Put biosynthesis (Hanfrey et al. 2001).

To determine whether the down-regulation of AtNAOD expression alters the biosynthesis of arginine or PAs, we measured the levels of $N^2$-acetylornithine, ornithine, arginine, Put, Spd and Spm in the leaves of atnaod and sil#17 plants (Fig. 6). No differences in the levels of $N^2$-acetylornithine, the substrate of the putative NAOD, were found in atnaod, sil#17 and...
wild-type plants (Fig. 6B). On the other hand, ornithine content was reduced by approximately 40% in both sil#17 and atnaod leaves, vs. wild-type leaves (Fig. 6B). Arginine accumulation was unchanged in atnaod, sil#17 and wild-type leaves (Fig. 6B). Taken together, these results demonstrate that AtNAOD regulates ornithine content in plant cells.

Although the arginine content was unaffected (Fig. 6B), Put levels in atnaod and sil#17 were 50% and 40% higher than in wild-type plants, respectively (Fig. 6C). No significant differences in Spd content were detected in either atnaod or sil#17 plants compared with wild-type plants (Fig. 6C). In contrast, Spm levels were reduced by 30% in both atnaod and sil#17 plants (Fig. 6C). Thus, the lack of AtNAOD function leads to the perturbation of ornithine levels with downstream effects on the levels of the diamine Put and the tetramine Spm.

**Discussion**

In angiosperms, flower and fruit development is a multistep process controlled by internal signals and environmental conditions. Fruit usually develops from the gynoecium, the female
Fig. 6 Evaluation of ornithine, arginine and free polyamine concentrations in loss-of-function AtNAOD plants. (A) Schematic representation of the arginine biosynthetic pathway and its association with polyamine biosynthesis. The terminal step for ornithine synthesis in the cyclic and linear pathways is reported in green and red, respectively. NAOGAT, N²-acetylornithine:N-acetylglutamate acetyltransferase; NAOD, acetylor

nithine deacetylase; ADC, arginine decarboxylase. *The gene encoding ornithine decarboxylase (ODC), which converts ornithine into putrescine, is apparently absent in A. thaliana (Hanfrey et al. 2001). (B) N²-Acetylornithine, ornithine and arginine concentrations measured in sil#17 and atnaod leaves compared with the wild type. (C) Free polyamine concentrations measured in sil#17 and atnaod leaves compared with the wild type. For each line, three biological replicates were prepared and the data are reported as average values ± SE (n = 3). For each biological replicate, the extractions and the quantifications were carried out twice (left and right sides of the bar graphs). Data were subjected to ANOVA followed by Tukey’s HSD test (P ≤ 0.05); for each metabolite, different letters indicate a statistically significant difference.
organ of the flower. The gynoeceum consists of modified leaves, which often are fused together into a single compound tubular vessel (pistil). The basal part of the pistil is the ovary in which the ovules are housed. After successful fertilization, the ovary starts to grow. The ovary wall becomes the pericarp of the fruit and the fertilized ovules develop into seeds. A critical step in fruit development is fruit setting, which represents the onset of ovary growth as a consequence of the suppression by fertilization of inhibitory factors (Fuentes and Smith 2009, Pandolfini et al. 2009). The ovules produce signals that control ovary growth after fertilization (Varga and Bruinsma 1986); these signals are principally hormonal. Auxin and gibberellin are positive signals for ovary growth that act in a hierarchical manner (Pandolfini et al. 2007, Serrani et al. 2008, Dorcey et al. 2009). After fertilization, a localized burst of auxin in the ovules activates gibberellin metabolism (Gillaspy et al. 1993, Vivian-Smith and Koluntow 1999, Wang et al. 2005, Goetz et al. 2006, Pandolfini et al. 2007). The concomitant action of the two hormones then promotes fruit growth (Wang et al. 2005, Goetz et al. 2007, Marti et al. 2007, Pandolfini et al. 2007, Serrani et al. 2008, Vriezen et al. 2008, de Jong et al. 2009, Sotelo-Silveira et al. 2014).


Parthenocarpic tomato plants, obtained by expressing an auxin-synthesizing transgene under the control of an ovary-specific promoter (DefH9::iaaM; Rotino et al. 1997, Ficcadenti et al. 1999, Pandolfini et al. 2002, Pandolfini et al. 2009), were used as an experimental model of fruit setting (Molesini et al. 2009a, Molesini et al. 2009b). Comparative transcriptomics of DefH9::iaaM parthenocarpic and wild-type flower buds yielded a reliable data set of genes putatively involved in fruit setting (Molesini et al. 2009a). For example, silencing SlAucsia, one of the differentially expressed genes identified, caused parthenocarpic fruit development, demonstrating its role as a key repressor of ovary growth (Molesini et al. 2009b). The same data set highlighted the importance of ornithine and PAs in the early phases of tomato fruit development. The genes encoding NAOD and acetylornithine aminotransferase, enzymes implicated in the ornithine/arginine biosynthetic pathway, were induced in parthenocarpic flower buds compared with wild-type buds; similarly, genes encoding ODC and spermidine synthase were up-regulated (Molesini et al. 2009a). These data are in accordance with current knowledge that the PAs may play a role in early fruit development in several plant species (Evans and Malmberg 1989, Egea-Cortines et al. 1993). High levels of free PAs were detected in tomato ovaries after pollination and in the early stages of the development of parthenocarpic ovaries after treatment with auxin or gibberellin (Heimer and Mizrahi 1982, Alabadi et al. 1996, Fos et al. 2003). Both in tomato and in Arabidopsis, the NAOD genes are highly expressed during flower/fruit development (Molesini et al. 2009a, Pandolfini et al. 2009, this study). In Arabidopsis, maximal expression occurs in female organs, especially in unpollinated ovules. A role for AtNAOD in controlling ovule functionality is supported by the phenotype of AtNAOD-down-regulated plants; these plants are defective for fruit development, as evidenced by a higher number of aborted siliques (≤0.5 cm in length) than in wild-type plants (Fig. 5). In addition, pollinated siliques (≥1.0 cm in length) were on average shorter than those in wild-type plants and contained fewer seeds (Fig. 5). Impairment in seed development was also reported by Frémont and collaborators (2013) in the Arabidopsis TUP5 mutant. TUP5 encodes an acetylornithine aminotransferase, which acts upstream of NAOD/NAOGAcT in arginine bio-synthesis (Fig. 6A). The same authors observed that the lack of arginine and a general lack of nitrogen storage molecules affect ovule and embryo development (Frémont et al. 2013). Our data indicate that AtNAOD contributes to the regulation of fruit set, possibly acting on ovule receptivity. It is interesting to note that in auxin-hypersynthesizing parthenocarpic tomato flower buds, where fruit setting occurs precociously (i.e. at pre-anthesis), the expression of SINAOA correlates with the onset of ovary/fruit growth (Molesini et al. 2009a, Pandolfini et al. 2009). Perturbations in early fruit development can involve changes in auxin and gibberellin metabolism and/or signaling (Wang et al. 2005, Molesini et al. 2009b). The sensitivity to these hormones was not affected in AtNAOD-down-regulated plants, suggesting that AtNAOD may act either independently of or downstream of auxin and gibberellin.

AtNAOD is annotated as a putative NAOD, leading us to predict that the observed phenotype is linked to alterations in ornithine/arginine concentrations or in PA content. For the first time, we showed here that AtNAOD contributes to the regulation of ornithine homeostasis. Although ornithine is produced in the glutamate to arginine via the action of NAOGAcT, the lack of AtNAOD severely lowered the ornithine content (Fig. 6), indicating that other enzymes involved in ornithine homeostasis do not compensate for the AtNAOD dysfunction.

The reduction in ornithine levels did not influence the arginine concentration but affected the levels of PAs (Fig. 6). This finding apparently contradicts the current knowledge that PAs in Arabidopsis are produced only via the ADC pathway. However, previous data have demonstrated that changes in the ornithine level do not directly affect arginine accumulation. For instance, Kalamaki and collaborators (2009) found that Arabidopsis plants overexpressing the N-acetyl-l-glutamate synthase of tomato (SINAGS1), which converts glutamate to N-acetylglutamate, accumulated ornithine but not arginine. Likewise, the overexpression of the mouse ODC in Arabidopsis seedlings did not influence arginine accumulation, although a large proportion of cellular ornithine was drained away by the activity of mouse ODC (Majumdar et al. 2013). Furthermore, manipulation of Arabidopsis arginase, an enzyme responsible for the conversion of arginine into ornithine and urea, did not affect the arginine accumulation, whereas the
relative arginine content (calculated as a percentage of total free amino acids) was significantly lower in both AtArginase-overexpressing and -knockout lines (Shi et al. 2013).

As several authors have pointed out, the cellular arginine level in higher plants is regulated by multiple mechanisms (Funck et al. 2008, Page et al. 2012, Shi and Chan 2013). The activity of biosynthetic enzymes and enzymes involved in downstream reactions (i.e. PA and nitric oxide production) as well as their cellular compartmentation (i.e. chloroplast and mitochondrion) contribute to arginine homeostasis (Slocum 2005, Lunn 2007, Funck et al. 2008). Hence, we cannot exclude that in atnaod and silenced plants, the arginine pool, specifically channeled in the PA biosynthetic pathway, might be affected.

In atnaod mutant and AtNAOD-silenced plants, we observed an altered PA profile with increased Put and decreased Spm concentrations compared with wild-type plants. These variations in PA levels cannot be simply explained by the depletion of the biosynthetic precursors in the glutamate → ornithine → arginine pathway. Besides biosynthesis, conjugation as well as degradation and transport regulate the cellular content of free PAs (Martin-Tanguy 2001, Bitrián et al. 2012). The accumulation of Put might be caused by a reduced conjugation and/or degradation as well as by the back-conversion of Spm into Spd and eventually into Put. This hypothesis would be in accordance with the diminished accumulation of Spm in atnaod and silenced plants.

The reproductive phenotypes observed in AtNAOD-downregulated plants can be reasonably connected to alterations in PA and/or ornithine metabolism. Experiments on tobacco plants showed that the overexpression of the ornithine cyclodeaminase (rolD) from Agrobacterium rhizogenes accelerated the induction of the flowering process and enhanced flower formation (Mauro et al. 1996, Trovato et al. 2001). Trovato and collaborators (2001) suggested that these phenotypes could be due either to ornithine depletions or to increased levels of citrulline, the product of rolD activity. Unfortunately, no data on PAs were reported in that study. On the other hand, numerous studies have demonstrated the involvement of PAs in different processes such as flowering, fruit setting and ripening (Carbonell and Navarro 1989, Imai et al. 2004, Liu et al. 2006, Handa and Mattoo 2010, Mattoo et al. 2010, Moschou et al. 2012). The mode of action of PAs has been correlated with their regulatory activity on senescence (Martin-Tanguy 2001, Mattoo et al. 2010, Moschou et al. 2012) and nitrogen (N) and carbon (C) balance (Handa and Mattoo 2010, Mattoo et al. 2010, Ren et al. 2012). In this regard, the diamine Put and the PAs Spd and Spm exert contrasting effects on cellular amino acid metabolism and sugar accumulation (Handa and Mattoo 2010, Mattoo et al. 2010). Data obtained by overexpressing mouse ODC in several plant systems revealed that Spd and Spm might act as positive regulators of amino acid metabolism, whereas a negative correlation between the Put level and the accumulation of various amino acids has been observed (Mattoo et al. 2010). On the other hand, glucose, fructose and sucrose exhibited a positive correlation with Put levels but were negatively associated with Spd and Spm (Mattoo et al. 2010). Based on these findings, it was hypothesized that the increase in Put level correlates with the condition of low metabolic activity, whereas the increase in Spd and Spm correlates with growth stimulation (Handa and Mattoo 2010, Mattoo et al. 2010, Moschou et al. 2012).

At the onset of flower and fruit formation, profound metabolic changes occur including source–sink relationships. Therefore, it is reasonable to speculate that the early reproductive phase transition and the impaired fruit setting observed in AtNAOD-down-regulated plants might be due to the effects of PAs on the control of N and C balance (Mattoo et al. 2006, Handa and Mattoo 2010, Mattoo et al. 2010, Ren et al. 2012). In this regard, a specific role for Spm in controlling ovary viability and fruit setting has been demonstrated (Carbonell and Navarro 1989).

Overall, the novel findings of the present work demonstrate a link between AtNAOD activity and the cellular levels of ornithine and PAs; they also provide evidence of a role for AtNAOD in flowering and in fruit setting, most probably through action at the level of the female organs. Further research will be necessary to resolve the biochemical activity of the AtNAOD protein in Arabidopsis.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana mutants and transgenic lines described were in the Columbia (Col-0) background. T-DNA insertion mutants SALK_108570 and SALK_046909 were identified from the SALK collection (Alonso et al. 2003) and the GABI-Kat collection (Kleinboeiling et al. 2012), respectively. The position of the T-DNA insertion and the allelic status were verified by PCR and sequencing of the T-DNA flanking regions.

All phenotypic characterizations were performed with homozygous plants. For in vitro assays, seeds were surface-sterilized, plated on half-strength MS (Murashige and Skoog 1962) medium (2.15 g l\(^{-1}\)) MS salts, 0.8% (w/v) plant agar, 1% (w/v) sucrose, pH 5.7, cold treated for 2–4 d at 4 °C, and then transferred to a growth chamber set at a constant temperature of 25 °C with a 16 h light/8 h dark photoperiod, with an average light intensity of 120 pmol m\(^{-2}\) s\(^{-1}\).

When plants were grown to maturity for reproductive phenotype evaluation, seeds were directly sown on soil in a greenhouse under a 16 h light/8 h dark photoperiod, at 24 °C and 20 °C, respectively.

Hormonal treatments

For quantitative analysis of root growth, seedlings were vertically grown on an MS agar plate. After 7 d, when the roots were approximately 1.5 cm in length, the seedlings were transferred to fresh MS plates supplemented with various concentrations of IAA (0.03 and 0.1 \(\mu\)M) and grown for 7 d.

For quantitative analysis of hypocotyl elongation, seedlings were grown for 7 d on an MS agar plate supplemented with various concentrations of GA\(_3\) (1, 10 and 100 \(\mu\)M).

For the measurement of root and hypocotyl elongation, the Image J program was used (Abramoff et al. 2004).

Plasmid construction and plant transformation

To create the transcriptional fusion construct AtNAOD::GUS, a 1,053 bp fragment upstream of the translation initiation codon was amplified from wild-type plants via PCR with the following forward (F) and reverse (R) primers: F, 5'-TGTGGTTTACCTTGGCACT-3' and R, 5'-TTTTTGTACACCGAGATTCTT-3'. The PCR fragment was cloned into a derivative of the pBluescript vector, which contains a 1,812 bp sequence of the GUS gene. To silence AtNAOD mRNA, we made a hairpin construct by assembling in the inverted orientation a 200 nucleotide long fragment corresponding to the coding sequence from nucleotides...
980 to 1,089 (+1 being the A of the ATG initiation codon) following the construct design described in Molesini et al. (2009b).

These constructs were introduced into Arabidopsis by Agrobacterium tumefaciens-mediated transformation (strain GV2260) by using the floral dip procedure (Zhang et al. 2006). Transgenic lines were selected after germination on MS medium containing 50 mg l−1 kanamycin.

GUS staining and light microscopy

Histochemical analysis of GUS activity in transgenic plants was performed as previously described by Jefferson and collaborators (1987), with some modifications. Briefly, plant tissues were immersed in the staining buffer [100 mM phosphate buffer, pH 7.5, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Triton X–100, 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolyl-[β-Glucuronide] at 37°C for 16–18 h. The staining solution was removed and the sample was cleared by rinsing several times in 70% (v/v) ethanol. Images were taken with a Leica DMRB microscope equipped with a DFC420C digital camera (Leica Microsystems).

Gene expression analysis by real-time qRT–PCR

Total RNA was purified from root, inflorescences, and leaves using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA was isolated from siliques using 20 (w/v) of high-sodium extraction buffer [100 mM Tris–HCl (pH 9.5), 10 mM EDTA (pH 8.0), 2% (v/v) lithium dodecyl sulfate, 0.6 M NaCl, 0.4 M trisodium citrate and 5% 2-mercaptoethanol] and following the method of Suzuki et al. (2004). cDNA synthesis, quantitative real-time PCR conditions and data analysis were as described in Molesini et al. (2012). The following forward (F) and reverse (R) primers were employed: for AtNAOD F, 5′-CGGGTGATGTCAGGTTGACT-3′; and R, 5′-TGAAAACGAGCCACGGTTT-3′; and for actin F, 5′-TGTTCTCTCTTGTACGCCAGT-3′; and R, 5′-CACGGAAGTCAACACGGAGGAGG-3′.

Quantification of amino acids and free PAs

The leaf material, collected from atnaod mutant, sil#17 and wild type plants, was immediately frozen in liquid nitrogen and stored at −80°C before analysis.

PAs and amino acids were extracted according to the following protocol. Briefly, 0.25 g of frozen samples was extracted in 1.5 ml of 75% (v/v) methanol containing 0.05% (v/v) trifluoroacetic acid (TFA). After homogenization, the samples were stirred for 40 min and centrifuged at 19,000 g for 10 min. The extracts were filtered through 0.2 μm polytetrafluoroethylene (PTFE) filters. For each line, three biological replicates were prepared. Each biological replicate was extracted twice, resulting in a total of 18 extracts; each extract was analyzed twice.

All the extracts were analyzed using reversed phase liquid chromatography coupled to a photodiode array detector and to an ion trap mass spectrometry (LC–PDA–MS) system. Such a system consisted of an ultra-performance liquid chromatography (UPLC) Dionex Ultimate 3000 model coupled to an LTQ XL mass spectrometer (Thermofisher Scientific). A 5 μl aliquot of sample was injected on a Luna C18 (100 × 2.0 mm, 2.5 μm particle size) column equipped with a SecurityGuard guard column (3.0 × 4.0 mm) from Phenomenex. The separations were carried out using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid, with a flow rate of 0.220 ml min−1.

The initial solvent composition consisted of 95% (v/v) of A and 5% (v/v) of B; increased linearly to 25% A and 75% B in 25 min and maintained for 1 min; returned to 95% of A in 1 min. The column was equilibrated to 95% A and 5% B for 11 min before the next injection. The analysis lasted for 38 min and the column temperature was set to 40°C. Mass spectra were obtained in positive ion mode over the range m/z 70–1,400. The capillary voltages were set at 9.95 V and the source temperature was 34°C. Quantitative determination of compounds was conducted by comparison with dose–response curves based on m/z data from authentic, distinct and appropriately diluted standard solutions of Spd, Spm, arginine, Put, ε-ornithine monohydrate and N-acetyl-ε-ornithine purchased from Sigma-Aldrich. Xcalibur software (Thermofisher Scientific) was used to control all instruments and for data acquisition and data analysis.

Statistical analyses

Statistical analyses of the data presented in Figs. 3, 4 and 5 were conducted using a Student’s t-test. The mean values ± SE are reported in all the figures.

Biochemical data showed in Fig. 6 were subjected to analysis of variance (ANOVA) performed with JMP software (SAS Institute) according to a completely randomized design. Means were compared by using Tukey’s HSD test (p ≤ 0.05).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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