Floral Transition and Nitric Oxide Emission During Flower Development in *Arabidopsis thaliana* is Affected in Nitrate Reductase-Deficient Plants

K. Seligman ¹, E. E. Saviani ¹, H. C. Oliveira ¹, C. A. F. Pinto-Maglio ² and I. Salgado ¹,*

¹ Departamento de Bioquímica, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas-UNICAMP, 13083-970, Campinas, SP, Brasil
² Laboratório de Citogenética, Centro de Pesquisa e Desenvolvimento de Recursos Genéticos Vegetais, CP 28, Instituto Agronômico de Campinas, 13020-902, Campinas, SP, Brasil

---

**The nitrate reductase (NR)-defective double mutant of *Arabidopsis thaliana* (*nia1 nia2*) has previously been shown to present a low endogenous content of NO in its leaves compared with the wild-type plants. In the present study, we analyzed the effect of NR mutation on floral induction and development of *A. thaliana*, as NO was recently described as one of the signals involved in the flowering process. The NO fluorescent probes diaminofluorescein-2 diacetate (DAF-2DA) and 1,2-diaminoanthraquinone (1,2-DAA) were used to localize NO production in situ by fluorescence microscopy in the floral structures of *A. thaliana* during floral development. Data were validated by incubating the intact tissues with DAF-2 and quantifying the DAF-2 triazole by fluorescence spectrometry. The results showed that NO is synthesized in specific cells and tissues in the floral structure and its production increases with floral development until anthesis. In the gynoecium, NO synthesis occurs only in differentiated stigmatic papillae of the floral bud, and, in the stamen, only anthers that are producing pollen grains synthesize NO. Sepals and petals do not show NO production. NR-deficient plants emitted less NO, although they showed the same pattern of NO emission in their floral organs. This mutant blossomed precociously when compared with wild-type plants, as measured by the increased caulinar/rosette leaf number and the decrease in the number of days to bolting and anthesis, and this phenotype seems to result from the markedly reduced NO levels in roots and leaves during vegetative growth. Overall, the results reveal a role for NR in the flowering process.**

**Keywords:** *Arabidopsis thaliana* — Diaminofluorescein — Floral induction — Flower development — Nitrate reductase (NR) — Nitric oxide (NO).

**Abbreviations:** cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide; 1,2-DAA, 1,2-diaminoanthraquinone; DAPI, 4',6-diamidino-2-phenylindole; DAF-2DA, diaminofluorescein-2 diacetate; DAF-2T, diaminofluorescein-2 triazole; DMSO, dimethylsulfoxide; MOPS, 3-(N-morpholino)propanesulfonic acid; NO, nitric oxide; NR, nitrate reductase.

---

**Introduction**

The free radical nitric oxide (NO) is involved in the regulation of a broad range of physiological and pathophysiological processes in animals, ranging from blood pressure and immune response to neural communication (Schmidt and Watler 1994). In the last decade, NO has also been implicated in the modulation of several physiological and developmental processes in plants, including flowering (see Lamattina et al. 2003, Desikan et al. 2004, Delledonne 2005, Simpson 2005, Salgado et al. 2004, Salgado et al. 2006, Neill et al. 2007).

The switch to flowering is a major developmental transition in the plant life cycle and has significant consequences for reproductive success of the species (Henderson and Dean 2004, Simpson and Dean 2002). Exogenous and endogenous signals, such as photoperiod, vernalization, gibberellins and the circadian clock, are known inducers of reproductive development (Mouradov et al. 2002, Simpson and Dean 2002). Recently, NO was revealed as one of the signals that controls the floral induction in *Arabidopsis*. He and collaborators (2004), when investigating the effect of NO on vegetative growth, showed that the application of the NO donor sodium nitroprusside resulted in a delay of flowering induction. Through genetic screening they identified *Arabidopsis* mutants with high or low endogenous NO levels, which delayed flowering or blossomed precociously, respectively, when compared with wild-type plants. Moreover, high levels of NO suppressed the *LFY* gene in the floral meristem and the *CO* gene for floral promotion, which positively affected flowering and, additionally, increased the expression of the *FLC* floral repressor gene. These results suggest a role for NO in suppressing floral transition by affecting the expression of genes involved in the regulation of the flowering process.

1-Arginine and nitrite are the major substrates for NO synthesis in various biological systems. In animals, the synthesis of NO during the oxidation of L-arginine to L-citrulline is well characterized and is accomplished by a family of nitric oxide synthase (NOS) enzymes (Stuehr 1997). NOS-like activity (Barroso et al. 1999, Ribeiro et al. 1997).
A. thaliana and a putative NOS-like enzyme have been identified in plants (Guo et al. 2003), although the function of this putative NOS-like enzyme in NO synthesis is controversial (Zemotjtel et al. 2006). In addition to L-arginine, nitrite is an important source of NO in plants and this activity was originally described as a side reaction of the nitrate reductase (NR) enzyme (Dean and Harper, 1986), the major activity of which is to reduce nitrate to nitrite during nitrogen assimilation. The nitrite-reducing activity of NR was later demonstrated by other groups (Yamasaki and Sakihama 2000, Neill et al. 2002, Rockel et al. 2002). In most of these reports, the nitrite-reducing activity of NR was substantiated by the inability of NR-deficient plants to produce NO. However, it was recently demonstrated that foliar extracts of Arabidopsis thaliana NR double mutant plants, nia1 nia2, present the same capacity to produce NO as wild-type plants when nitrite is exogenously supplied (Modolo et al. 2005). These mutant plants have lower endogenous contents of nitrite (Modolo et al. 2005) and L-arginine (Modolo et al. 2006) in their leaves, which may account for their decreased capability for NO synthesis. These results suggested that the NR enzyme, in addition to its nitrite-reducing activity, also has an important role in generating substrates for NO synthesis, and an imbalance in its endogenous content may affect NO-mediated processes. Indeed, the lower endogenous NO content of NR-deficient A. thaliana plants leaves them susceptible to pathogen attack (Modolo et al. 2006), as plant defense response to pathogens is known to be mediated by NO (Delledonne et al. 1998, Modolo et al. 2002). In accordance with these findings, pathogen signals were shown to induce expression of NR genes in potato tubers (Yamamoto et al. 2003), and silencing of NR genes significantly decreased elicitin-induced NO production in Nicotiana benthamiana (Yamamoto-Katou et al. 2006).

Direct measurement of NO is a difficult task because of its low concentrations and the extremely short-life of this radical in biological systems. A series of diaminofluoresceins, indicator probes that are very sensitive (detection limits of 5 nM) and non-cytotoxic, were originally developed for monitoring NO production in neuronal cells (Kojima et al. 1998). These fluorescent probes have been used widely for NO monitoring in plant tissues (Pedroso et al. 2000, Pagnussat et al. 2002, Neill et al. 2002, Corpas et al. 2004, Prado et al. 2004, Modolo et al. 2006, Zhao et al. 2007). Frequently, tissues are treated with the esterified form of the probe, diaminofluorescein-2 diacetate (DAF-2DA), which diffuses into cells where esterases hydrolyze the diacetate residues, thereby trapping DAF-2 within the intracellular space. In the presence of oxygen, NO or the NO-derived species nitrosate DAF-2 to produce the highly fluorescent DAF-2 triazole (DAF-2T).

Despite the high sensitivity of the diaminofluoresceins for NO, its use for monitoring NO in plants has received criticism (Stohr and Stremlau 2006) due to the observation that diaminofluoresceins also react with dehydroascorbic and ascorbic acid to generate new compounds that have fluorescence emission profiles similar to DAF-2T (Zhang et al. 2002). Considering that dehydroascorbic and ascorbic acid are common antioxidants, these cross-reactions may result in confounding interpretations when the diacetate form of the probe is used to localize in situ NO production. However, Ye and collaborators (2004) have shown that by incubating sample tissues directly with DAF-2 these side effects may be avoided because NO, in contrast to the interfering molecules, is a gas and can diffuse out of the tissue and react with DAF-2.

In the present study, we compared the sites of NO production during flower development and the floral induction of wild-type A. thaliana with that of the nia1 nia2 mutant plants. We used DAF-2DA and 1,2-DAA to localize in situ NO production in the floral buds of A. thaliana, and the results were validated by quantifying NO emitted from different parts of the plant using the free form of the diaminofluorescein probe. We discuss the importance of NR-dependent NO synthesis in the flowering process.

**Results**

**NO production in Arabidopsis thaliana floral structures localized with DAF-2DA**

NO production in wild-type A. thaliana floral buds in stage 12 of development was analyzed by fluorescence microscopy of individual floral structures—gynoecium, stamens, sepals and petals—previously treated with the NO fluorescent probe DAF-2DA. Stage 12 of floral development represents the last stage that precedes bud opening, and corresponds to that in which stigmatic papillae in the gynoecium are fully developed and stamens begin to produce pollen grains (Smyth et al. 1990). As shown in Fig. 1A, in gynoecium previously treated with DAF-2DA, only the stigmatic papillae showed high levels of NO emission, indicated by the green fluorescence of DAF-2T. At this stage of development, NO production appears to be restricted to certain regions of the stigmatic cell (Fig. 1B). In the stamens, NO production was detected in the gametes (Fig. 1C, D) that, in this phase of development, have already undergone meiosis and the resulting microspore cells are being differentiated to pollen grains. When gynoecium was previously treated with the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (cPTIO), DAF-2T fluorescence in stigmatic papillae was blocked (Fig. 1E), indicating that it corresponded to NO emission. Green fluorescence emission in the anthers was also blocked by
cPTIO (Fig. 1F). Sepals and petals exhibited no green fluorescence that would be indicative of NO production but only red fluorescence that corresponds to the chlorophyll autofluorescence at the wavelength analyzed (Fig. 1G, H). No fluorescent signals were detected in the controls where the floral structures were treated with water instead of DAF-2DA (not shown).

Floral buds of nia1 nia2 A. thaliana mutant plants at stage 12 of development were also imaged for NO after treatment with DAF-2DA. As seen in the floral structures of the wild-type plant, in this mutant, DAF-2T fluorescence was also restricted to the stigmatic papillae of the gynoecium, and in the stamens, DAF-2T fluorescence was only detected in anthers producing pollen grains (see below).

NO production during flower development localized with DAF-2DA

Fig. 2 shows NO fluorescent images detected in the gynoecium and anthers of the nia1 nia2 mutant at different stages of floral development. At stage 8, which represents
the beginning of the late phase of flower development and, after which most of the cellular differentiation takes place (Smyth et al. 1990), NO emission in the gynoecium was restricted to the site where the stigmatic papillae will develop (Fig. 2A). Significant detection of NO emission takes place only at stage 11 of floral development, in the newly differentiated stigmatic papillae that appear at the top of the stiletto (Fig. 2B). At stage 12, fully developed stigmatic papillae continue producing NO (Fig. 2C). At stage 13, when the bud opens and petals are visible, NO emission is decreased at the stigmatic papillae (Fig. 2D).

In stamens of nia1 nia2 mutants at stage 8 of development, when they have already begun their differentiation but are not yet producing pollen grains, there is no sign of NO emission (Fig. 2E). At this stage, stamens present only red fluorescence in the wavelength analyzed, which corresponds to the chlorophyll autofluorescence. At stage 11 of stamen development (Fig. 2F) NO production is discretely seen in the microspores. At stages 12 and 13, NO production is clearly seen in the microspores and forming pollen grains (Fig. 2G, H).

Floral structures of wild-type plants at different stages of floral development were also analyzed for NO imaging after treatment with DAF-2DA. The results were similar to those observed in nia1 nia2 mutants,
with maximal NO emission at stages 11 and 12 in the gynoecium and pollen grains (as showed in Fig. 1), indicating that the sites of NO emission during flower development are not influenced by NR double mutation.

**Fig. 3**  In situ NO production in wild-type *A. thaliana* stigmatic papillae and microspores detected with 1,2-DAA. Floral structures were treated with 1,2-DAA, and NO was detected by the red fluorescence of the 1,2-DAA triazole. (A, C, E) Gynoecium and (B, D, F) stamen at stages 11 (A, B), 12 (C, D) and 13 (E, F) of floral development treated with 1,2-DAA. (G, H) Gynoecium and stamen, respectively, at stage 12 of development treated with cPTIO and 1,2-DAA (control). (I, K) Images of pollen grains treated with 1,2-DAA and (J, L) the corresponding images of blue fluorescent nuclear staining with DAPI. v, vegetative nucleus; g, generative nucleus.

NO production in floral structures localized with 1,2-DAA

NO emission during floral development of *A. thaliana* was checked by using the NO fluorescent probe 1,2-diaminoanthraquinone (1,2-DAA). 1,2-DAA is considered...
to be more specific than DAF-2DA for in situ NO visualization, as it reacts selectively with NO (Heiduschka and Thanos 1998, Dacres and Narayanaswamy 2005). The pattern of NO emission did not differ between both genotypes, and fluorescent images obtained for the wild-type floral structures are shown in Fig. 3. At stage 11 of development the gynoecium showed intense red fluorescence emission, indicative of NO, at the stigmatic papillae (Fig. 3A), and in the stamens it is possible to see that the newly formed microspores are not yet producing NO (Fig. 3B). At stage 12, stigmatic papillae continue producing NO (Fig. 3C) and in the anthers NO is emitted by the mature microspores (Fig. 3D). At stage 13, NO emission in stigmatic papillae is not seen (Fig. 3E), and in the stamen (Fig. 3F) it is possible to see tetrads with almost no NO production, while intense NO synthesis is seen in mature microspores. NO emission was not detected when gynoecium and anthers, at stage 12 of development, were treated with cPTIO and 1,2-DAA (Fig. 3G and H, respectively), indicating that the red fluorescence emission detected in the stigmatic papillae and in the microspores can be attributed to NO. In order to follow NO production during gametogenesis, stamens were treated with 1,2-DAA and 4',6-diamidino-2-phenylindole (DAPI), a specific fluorescent marker of the nucleus. As shown in Fig. 3I–L, NO production is maximal in the uninucleate microspore and almost absent in the tri-nucleated pollen grains, where the two more brightly stained small generative nuclei and the larger and more diffusely stained vegetative nucleus are seen by the blue fluorescence of DAPI.

Quantification of NO emission from floral buds and vegetative tissues using DAF-2

DAF-2 was used to quantify NO emitted from the floral structures by adapting the procedure described by Ye and collaborators (2004). Intact structures were incubated with DAF-2. During the incubation period, NO emitted from the tissue can be released into the solution and trapped by the probe (Ye et al. 2004). Scanning emission fluorescence of supernatants from wild-type floral buds showed peak emission at 515 nm, upon excitation at 495 nm, characteristic of DAF-2T emission fluorescence (Fig. 4A). Supernatant solutions from floral buds incubated with DAF-2DA, instead of DAF-2, showed no fluorescence emission in this wavelength range (Fig. 4A) confirming that the esterified form of the probe does not detect NO released from the tissues. Fig. 4B shows DAF-2T emission fluorescence at 515 nm from supernatant solutions of floral buds of both genotypes at different stages of flower development, when previously incubated with DAF-2. As can be seen, much larger amounts of NO are emitted from wild-type floral buds at stage 11 (15.07 ± 0.65 nmol g⁻¹ h⁻¹) than at stages 8 (6.55 ± 0.61 nmol g⁻¹ h⁻¹) and 13 (5.18 ± 0.53 nmol g⁻¹ h⁻¹) of floral development. In nia1 nia2 floral buds, estimated NO emission at stages 8, 11 and 13 reached 4.21 ± 0.54, 11.04 ± 0.9 and 2.75 ± 0.41 nmol g⁻¹ h⁻¹, respectively. These results corroborated those obtained through in situ NO localization using DAF-2DA (Fig. 2) and 1,2-DAA (Fig. 3), showing maximal synthesis of NO just before flower opening. Additionally, it was possible to show that floral buds of wild-type plants emitted more NO than nia1 nia2 mutant plants; at stage 11 of floral development, when NO is maximally emitted, production of the radical was reduced by 26.74% in the NR-deficient plant. These results showed that differences in NO emission by floral buds that could not be detected between the two genotypes by in situ NO localization using DAF-2DA (Fig. 2) and 1,2-DAA (Fig. 3), showing maximal synthesis of NO just before flower opening.
leaves and 1.74/C6/mutation in plants. The overall results showed that NR double increased from 0.17 in wild-type to 0.23 in ratio of cauline/rosette leaf number at the day of anthesis respectively, at the day of anthesis (Fig. 5). Thus, the wild-type plants (40.87/C6/mutant, which represents an important difference nia2 was anticipated by 5.4 and 5.3, respectively, in the nia2 mutants (35.56/C6). Additionally, on average, wild-type and a life cycle of about 6 weeks under the used growth conditions. Moreover, in plants grown under our experimental conditions (12 h light/12 h dark), bolting in the wild-type cultivar occurred 37.09 ± 0.78 d after seeding, while in nia1 nia2 mutant plants bolting occurred earlier, at 31.69 ± 0.31 d after seeding. Similarly, anthesis, the opening of the floral bud, occurred earlier in nia1 nia2 mutants (35.56 ± 0.31 d after seeding) compared with wild-type plants (40.87 ± 0.99 d after seeding). Thus, on average, the number of days to bolting and anthesis was anticipated by 5.4 and 5.3, respectively, in the nia1 nia2 mutant, which represents an important difference in the timing of floral induction, for a species that has a life cycle of about 6 weeks under the used growth conditions. Additionally, on average, wild-type and nia1 nia2 presented 10.26 ± 0.28 and 8.56 ± 0.32 rosette leaves and 1.74 ± 0.18 and 1.97 ± 0.12 cauline leaves, respectively, at the day of anthesis (Fig. 5). Thus, the ratio of cauline/rosette leaf number at the day of anthesis increased from 0.17 in wild-type to 0.23 in nia1 nia2 plants. The overall results showed that NR double mutation in A. thaliana resulted in an earlier flowering

**Nitrate reductase activity**

NR activity was evaluated in floral buds, leaves and roots by monitoring NO3− reduction to NO2−. While leaves and roots of wild-type plants had an NR activity of 646.57 ± 17.47 and 579.30 ± 9.30 pmol NO2− min−1 mg−1, respectively, in floral buds, NR activity, if any, was very low. NR activity in floral buds at stage 8, when detected, was in the range of 11.82 ± 5.46 pmol NO2− min−1 mg−1, and at stage 11 and in opened flowers (stage 13 and later) it was not detected. As expected, NR activity was not measurable in any of the extracts of nia1 nia2 double mutants (floral buds, leaves or roots), confirming the original description by Wilkinson and Crawford (1993) of the extremely low NR activity of the NR double-deficient A. thaliana plants.

**Effect of NR mutation on floral induction**

As shown in Fig. 5, in plants grown under our experimental conditions (12 h light/12 h dark), bolting in the wild-type cultivar occurred 37.09 ± 0.78 d after seeding, while in nia1 nia2 mutant plants bolting occurred earlier, at 31.69 ± 0.31 d after seeding. Similarly, anthesis, the opening of the floral bud, occurred earlier in nia1 nia2 mutants (35.56 ± 0.31 d after seeding) compared with wild-type plants (40.87 ± 0.99 d after seeding). Thus, on average, the number of days to bolting and anthesis was anticipated by 5.4 and 5.3, respectively, in the nia1 nia2 mutant, which represents an important difference in the timing of floral induction, for a species that has a life cycle of about 6 weeks under the used growth conditions. Additionally, on average, wild-type and nia1 nia2 presented 10.26 ± 0.28 and 8.56 ± 0.32 rosette leaves and 1.74 ± 0.18 and 1.97 ± 0.12 cauline leaves, respectively, at the day of anthesis (Fig. 5). Thus, the ratio of cauline/rosette leaf number at the day of anthesis increased from 0.17 in wild-type to 0.23 in nia1 nia2 plants. The overall results showed that NR double mutation in A. thaliana resulted in an earlier flowering

**Figure 5**

Floral induction parameters of wild-type and nia1 nia2 A. thaliana plants. The parameters analyzed were the number of days to bolting (when the plant had a bolt of 1 cm), the number of days to anthesis (bud opening) and the number of rosette and cauline leaves (counted at the day of anthesis), as indicated. Data represent the mean of three independent experiments, with n = 35–40. The error bars represent ±SE.

**Discussion**

In the present study, by using two fluorescent probes, we localized the sites of NO production in floral structures of wild-type A. thaliana and showed that NO is emitted by specific cell types of the gynoecium and stamens, while sepalas and petals do not show any significant NO emission. Stigmatic papillae and mature microspores are prone to NO emission, indicating that only structures directly involved with the reproductive system synthesize this signaling radical. Moreover, by following NO production during the late stages of flower development we could show that maximal NO production coincides with the morphological differentiation of the reproductive organ, i.e. the stigmatic papillae differentiation and microspore maturation. The observation that NO is produced in specific structures in the plant reproductive system and at a developmental stage that precedes pollination suggests a role for NO in plant reproduction. Our data corroborate previous observations in Senecio and Arabidopsis stigmas, where a decrease in NO emission was observed at advanced stages of flower development while developing pollen grains presented an intense NO emission when followed with DAF-2DA (McInnis et al. 2006).

By comparing NO production in floral structures of wild-type plants with that of the NR double-deficient mutant plants we observed that the sites and stages where NO is maximally produced in floral structures of A. thaliana are not affected by NR mutation. In order to quantify and check the specificity of the in situ NO localization when treating floral structures of A. thaliana with DAF-2DA,
we measured DAF-2T fluorescence after incubating floral structures with the free form of the probe. When DAF-2 is directly used to quantify NO emission, interfering molecules such as dehydroascorbic and ascorbic acids can be avoided (Ye et al. 2004). The results obtained validated the experiments of in situ NO localization using DAF-2DA and 1,2-DAA showing that total NO emission by floral structures decreases with floral senescence. Additionally, NR-deficient plants were shown to produce less NO in their floral structures when compared with wild-type plants. The reduction of approximately 26% in NO emission by floral structures with the free form of the probe. When DAF-2 is directly used to quantify NO emission, interfering molecules such as dehydroascorbic and ascorbic acids can be avoided (Ye et al. 2004). The results obtained validated the experiments of in situ NO localization using DAF-2DA and 1,2-DAA showing that total NO emission by floral structures decreases with floral senescence. Additionally, NR-deficient plants were shown to produce less NO in their floral structures when compared with wild-type plants. The reduction of approximately 26% in NO emission by floral structures with the free form of the probe. When DAF-2 is directly used to quantify NO emission, interfering molecules such as dehydroascorbic and ascorbic acids can be avoided (Ye et al. 2004). The results obtained validated the experiments of in situ NO localization using DAF-2DA and 1,2-DAA showing that total NO emission by floral structures decreases with floral senescence. Additionally, NR-deficient plants were shown to produce less NO in their floral structures when compared with wild-type plants. The reduction of approximately 26% in NO emission by floral structures with the free form of the probe. When DAF-2 is directly used to quantify NO emission, interfering molecules such as dehydroascorbic and ascorbic acids can be avoided (Ye et al. 2004). The results obtained validated the experiments of in situ NO localization using DAF-2DA and 1,2-DAA showing that total NO emission by floral structures decreases with floral senescence. Additionally, NR-deficient plants were shown to produce less NO in their floral structures when compared with wild-type plants. 

Overall, the present results suggest that NO production during flower development and floral induction is affected in nia1 nia2 mutant plants, revealing a role for NR in flowering control and plant reproduction.

Materials and Methods

Plant material and culture conditions

The assays were conducted with wild-type plants of A. thaliana L. ecotype Columbia-0 and with nia1 nia2 double-mutant plants of the structural genes of NR, developed by Wilkinson and Crawford (1993). The seeds were sterilized with sodium hypochloride (0.3%) for 10 min, and the plants cultivated in pots with vermiculite:perlita (1:1). Wild-type plants were irrigated with half-strength Murashige and Skoog (1962) liquid medium without the addition of glucose and glycine. The nia1 nia2 plants were cultured in the medium described by Wilkinson and Crawford (1991) with reduced amounts of nitrate and ammonia as the main nitrogen sources. The plants were cultivated under controlled conditions in growth chambers at a relative humidity of 100%, 24°C and a photoperiod of 12 h light and 12 h dark. Floral buttons at stages 8, 11, 12 and 13 of development (Smyth et al. 1990) were used in the experiments.

Nitrate reductase activity

Floral buds, leaves and roots were used to determine the NR activity, as described by Su et al. (1996), with some modifications. Arabidopsis thaliana tissues (150 mg) were ground with a mortar and pestle in liquid N2 and then homogenized in 1 ml of cooled extraction buffer, that contained 50 mM 3-(N-morpholino) propesulfonic acid (MOPS), pH 7.4, 1 mM EDTA, 5 µM FAD and 7 mM cysteine. The mixture was centrifuged at 10,000 x g for 10 min at 4°C. An equal volume of assay buffer, composed of 50 mM MOPS, pH 7.4, 10 mM NaN3, 1 mM EDTA and 1 mM NADH, was added to the resulting supernatant and the mixture incubated in the dark at room temperature. After 60 min, the reaction was stopped by the addition of the Griess reagent, and nitrite production was quantified at 540 nm (Green et al. 1982). The NR activity assay was done in the dark to avoid direct nitrate conversion into ammonium (Dembinski et al. 1996).

NO detection by fluorescence microscopy

Samples of A. thaliana floral buds were collected and the floral structures were separated (gynoecium, stamen, sepals and petals) with the aid of a stereomicroscope, and were immediately treated for the visualization of NO using DAF-2DA (Calbiochem-Novabiochem, San Diego, CA, USA). Samples of each structure were incubated with 10 µM DAF-2DA dissolved in 0.1 M MOPS buffer (pH 7.2) for 30 min, under agitation, at 25°C in darkness. After this period the solution was removed and the floral structures were washed once with distilled water. Alternatively, gynoecium and stamen were treated with 50 µM 1,2-DAA (Calbiochem-Novabiochem), prepared in 100% dimethylsulfoxide (DMSO), instead of using DAF-2DA. For the negative controls, the floral structures were submitted to the same conditions, using deionized water instead of the fluorescent probe. To test the specificity of the fluorescent probes, the samples were also treated with the NO scavenger, ePTIO (Calbiochem), at 1 mM for 30 min before incubation with the fluorescent probe. After the treatments, the samples were mounted on glass coverslips in Vectashield (which delays the burning of the fluorescent probe).
containing DAPI (for DNA staining) and analyzed using a Olympus BX50 fluorescence microscope, equipped with an Olympus CCD (charge-coupled device) digital chamber Q-Color, with refrigeration. For analysis of treated samples, the following Olympus filters were used: U-MNB2 (excitation at 450 nm and emission at 570 nm) for analysis of the DAF-2DA-treated samples (Kojima et al. 1998); U-N41005 (excitation at 535–550 nm and emission 645–675 nm) for 1,2-DAA-treated samples (Halbach 2003) and U-MWU for DAPI (excitation at 372 nm and emission at 456 nm) (Lakowicz 1999). Images were acquired using the computer software Image-Pro Plus, version 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA) and processed using the software Corel Photo Paint. Images shown in the figures are representative of at least five (DAF-2DA) and three (1,2-DAA) replicates per treatment.

Spectrofluorimetric assays

Quantification of NO emission was carried out by incubating plant tissues directly with DAF-2 in order to eliminate the interference of intracellular compounds which may react with NO (Zhang et al. 2002), and these assays were based on the method developed by Ye and collaborators (2004). Floral buds, roots and leaves were incubated for 1 h at 25°C, in darkness, with 75 µM DAF-2 (or DAF-2DA for control) prepared in 0.1 M phosphate buffer (pH 7.2). Reaction mixtures from floral buds were then frozen and incubated for an additional period of 24 h at –80°C, to increase total NO emission, according to Ye and collaborators (2004). As this additional step did not significantly increase NO emission from floral buds (not shown), it was omitted in the assays with roots and leaves. The same buffer was then added to the reaction mixture to dilute the probe to a final concentration of 10 µM, and the suspension was centrifuged at 7,500 × g to precipitate the tissues. DAF-2T fluorescence of the supernatant was analyzed in a Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo, Japan). Emission fluorescence was scanned from 500 to 560 nm upon excitation at 495 nm. Estimation of NO concentration was calculated from a calibration curve using the NO donor DEA-NONOate, 2-(N,N-diethylamine)-diazenolate-2-oxide (Keefe et al. 1996).

Measurement of flowering time

Floral induction parameters were analyzed by counting the days to bolting, the days to anthesis, and rosette and caulinar leaf number at day of anthesis. The time of bolting was determined as the day when the plant had a bolt of 1 cm. Parameters of 35–40 plants were measured, in three different experiments.

Funding

Fundação de Amparo à Pesquisa do Estado de São Paulo (05/54246-4); Conselho Nacional de Desenvolvimento Científico e Tecnológico (to I.S.).

Acknowledgments

The authors thank Dr. Eneida de Paula for the use of the spectrofluorometer.

References


Modolo, L.V., Augusto, O., Almeida, I.M., Magalhães, J.R. and Salgado, I. (2005) Nitrite as the major source of nitric oxide production by...


(Received May 7, 2008; Accepted June 4, 2008)