Nitrogen dioxide accelerates flowering without changing the number of leaves at flowering in *Arabidopsis thaliana*

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A negative correlation has consistently been reported between the change in flowering time and the change in leaf number at flowering in response to environmental stimuli, such as the application of exogenous compounds, cold temperature, day length and light quality treatments in *Arabidopsis thaliana* (Arabidopsis). However, we show here that the application of exogenous nitrogen dioxide (NO$_2$) did not change the number of rosette leaves at flowering, but actually accelerated flowering in Arabidopsis. Furthermore, NO$_2$ treatment was found to increase the rate of leaf appearance. Based on these results, reaching the maximum rosette leaf number earlier in response to NO$_2$ treatment resulted in earlier flowering relative to controls.

Similar to other herbaceous plant species, the time to flowering or timing for transition from the vegetative to reproductive phase is a critical factor determining vegetative biomass yield in *Arabidopsis thaliana* (Arabidopsis). Late flowering contributes to increased vegetative biomass yield in Arabidopsis and other species.[1-4] Flowering time increases or decreases as a function of environmental stimuli and Arabidopsis genotype, and the relationship between flowering time and number of rosette leaves or combined number of rosette and cauline leaves (i.e., leaf number) at flowering, which reflects the final vegetative biomass, is not always proportional.[5-7] However, to our knowledge the correlation between change in flowering time and change in leaf number in response to various environmental stimuli is always negative in Arabidopsis. For example, application of exogenous nitric oxide (NO),[8] glucos,[9] sucrose[10] and gibberellic acid (GA)[11] increases leaf number but delays flowering. Vernalization treatment reduces leaf number but accelerates flowering.[12,13] In addition, short day treatment increases leaf number but delays flowering.[14] Quality and intensity of light affects leaf number and negatively affects flowering time.[11,14] Moreover, leaf number is commonly used as a standard indicator of flowering time; the fewer the leaves, the faster is flowering.[15,16]

In contrast to these stimuli, atmospheric nitrogen dioxide (NO$_2$) increases shoot biomass and organ size, accompanied by acceleration of flowering, as reported previously.[17-19] Furthermore, an increase in shoot biomass following NO$_2$ treatment was correlated with leaf size rather than leaf number.[17] Thus, treatment with exogenous NO$_2$ is unique in terms of the control of

vegetative and reproductive growth compared to other various environmental stimuli, as described above. Exploring this unique effect of NO$_2$ is important to increase our understanding of NO$_2$ physiology and of the mechanistic interactions of vegetative growth with flowering. In this report, we investigated the relationship between flowering time and leaf number in response to NO$_2$ treatment. Arabidopsis plants were grown and subjected to NO$_2$ treatment, as reported previously[17] (see supplementary material). The number of days after sowing when flower bolts were 1 cm in length was used as a measure of flowering time.[20]

Table 1 summarizes the effects of NO$_2$ treatment on flowering time and shoot biomass yield in 3 Arabidopsis accessions; namely, C24, Columbia (Col-0) and *Landsberg erecta* (Ler) obtained in our present (flowering time of Ler) and previous reports.[17-18] Ler significantly accelerated flowering by 2.1 days and increased shoot biomass yield following NO$_2$ treatment, as has been observed for 2 other accessions. NO$_2$ treatment increased shoot biomass accompanied by acceleration of flowering time in all 3 accessions (**Table 1**).

Despite the acceleration of flowering, NO$_2$ treatment did not change the number of rosette leaves per plant at flowering or the maximum rosette leaf number (**Table 2**). Furthermore, NO$_2$ treatment significantly increased, by about 20%, the rate of leaf appearance (RLA) or the inverse of plastochron (**Table 2**).

Leaf number is commonly used as a standard indicator of flowering time in Arabidopsis.[15,16] This means that the number of rosette leaves per plant at flowering, or the maximum rosette leaf number on plants of an Arabidopsis genotype grown under a

Table 1. Application of exogenous nitrogen dioxide (NO_2) accelerated flowering and increased shoot biomass in *Arabidopsis thaliana* accessions C24, Columbia (Col) and *Landsberg erecta* (Ler)

Accession	NO_2 concentration (ppb)	Average flowering time (days) $\pm$ SD (*n*)	Average dry wt. of shoots (mg per plant) $\pm$ SD (*n*)
C24	0	42 $\pm$ 3 (20)	25.2 $\pm$ 6.8 (4)
	50	40 $\pm$ 3 (20) *	68.5 $\pm$ 9.3 (10) ***
Col	0	40 $\pm$ 3 (17)	14.3 $\pm$ 2.5 (5)
	50	34 $\pm$ 1 (26) ***	24.2 $\pm$ 5.5 (5) *
Ler	0	29.6 $\pm$ 1.5 (19)	11.3 $\pm$ 2.6 (5)
	50	27.5 $\pm$ 2.1 (22)***	28.2 $\pm$ 2.3 (5) ***

All data, excluding average flowering time of Ler, were obtained from our previous studies.[17,18] Plants were harvested at 5, 4, and 4 weeks after sowing for C24, Col, and Ler, respectively (see supplementary material). The number of days after sowing when flower bolts were 1 cm in length was used as a measure of flowering time.[20] Statistical significance was assessed using Dunnett's *t*-test for dry wt. and Student's *t*-test for flowering time: *, P $<$ 0.05; ***, P $<$ 0.001. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

given set of environmental conditions (excluding NO_2 exposure), is considered a constant. However, our present finding that the number of rosette leaves per plant at flowering was similar between $+NO_2$-treated plants and $-NO_2$ control plants (**Table 2**) indicated that NO_2 treatment did not change rosette leaf number (**Table 2**). Thus, rosette leaf number cannot be used as an indicator of flowering in response to NO_2 treatment.

Nonetheless, our finding suggested that the number of rosette leaves on $+NO_2$-treated and $-NO_2$ control plants triggered flowering, and that the number of leaves that triggered flowering was identical in both plants. The NO_2 treatment did not change rosette leaf number at flowering but influenced the RLA, with that of $+NO_2$-treated plants being higher than that of $-NO_2$ control plants (**Table 2**). Thus, the combination of these 2 experimental observations, identical maximum leaf number and shortened plastochron, is a likely cause of the earlier flowering in response to NO_2 treatment.

Among the 4 or 5 major pathways for Arabidopsis flowering,[21,22] NO_2-induced early flowering is likely associated with the autonomous and aging endogenous pathways, both of which function independently of environmental factors and are related to the developmental stage of the plant. However, quantitative real-time PCR analysis of mRNA levels in Col-0 revealed that the expression of genes such as *FLC* and *MIR156*, which are reported to be involved in these endogenous pathways,[21,22] did not change in response to NO_2 treatment (Takahashi et al., unpublished results). Identification of floral genes involved in NO_2-induced flowering control requires further investigation.

It has been reported recently that disruption of a gene encoding the protein GIGANTUS1, a member of the transducin/WD40 protein superfamily, results in lower leaf number and earlier flowering, and higher shoot biomass accumulation)

compared to wild-type Arabidopsis controls.[23] The involvement of the *GIGANTUS1* gene in NO_2-induced vegetative growth and early flowering requires further study.

We reported previously that NO_2-induced increases in shoot biomass and organ size are attributable to NO_2-induced increases in cell proliferation and enlargement.[17] The genes involved in increased organ size and biomass upon ectopic overexpression (for positive regulators) or down-regulation (for negative regulators) have been referred to recently as intrinsic yield genes (IYGs); these genes have been investigated using Arabidopsis as a model plant (reviewed by Gonzalez et al.[24]). Investigations using overexpressors of positive regulators or transgenic plants in which negative regulators were suppressed have shown that multiple pathways independently converge to control organ size in Arabidopsis.[24] A total of 23 IYGs—including 9, 11 and 3 genes that stimulate cell proliferation, enlargement, and both cell proliferation and enlargement, respectively—were chosen and analyzed using quantitative real-time PCR for their average transcript expression levels in young (leaves 21–25), middle (leaves 12–20), and old (leaves 1–11) leaves of 5-week-old $+NO_2$-treated plants and $-NO_2$ control plants. No single gene was consistently significantly up- or down-regulated. However, NO_2-induced expression of different sets of these genes was dependent on the leaf developmental stage.[17]

Besides IYGs, a group of genes that strongly affected the rate of leaf production and plastochron identified in Arabidopsis, rice, and maize such as *PHYB*, gibberellin biosynthesis and sensitivity genes, *PGM*, *PLASTOCHRON1*, *CYP78A5*, *CYP78A7*, *AMP1*, *PLA3*, *TE1*, *PLA2*, and *SE* (see Méndez-Vigo[25]) are likely involved in NO_2-induced stimulation of vegetative growth and flowering. Interestingly, involvement of some of these genes in flowering-related traits, including flowering time, has been

Table 2. Number of rosette leaves per plant at flowering, and average rate of leaf appearance of Arabidopsis (Col-0) plants treated with 0 or 50 ppb NO_2

NO_2 (ppb)	Number of rosette leaves per plant[a]	Average rate of leaf appearance[b] (leaves / day)
0	21.2 $\pm$ 2.7 (19)	0.53 $\pm$ 0.07 (19)
50	21.5 $\pm$ 3.1 (20)	0.63 $\pm$ 0.09 (20) ***

Data represent means $\pm$ SD (*n*). Plants were grown and subjected to NO_2 treatment as described in the supplementary material. The number of days after sowing when flower bolts were 1 cm in length was used as a measure of flowering time.[20] [a] Number of rosette leaves per plants were counted at flowering. [b] Number of rosette leaves at flowering was divided by the number of days after sowing at flowering (40 and 34 days for plants treated with 0 and 50 ppb NO_2, respectively[17], see **Table 1**). ***, P $<$ 0.001. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

suggested.[25] More recently, epistatic interactions of the genes involved in circadian rhythm and starch metabolism, leading to growth vigor and increased biomass accumulation, have been reported.[1,26-28] The role of these genes, including their interactions in NO_2-induced acceleration of both shoot biomass accumulation and flowering, remains unknown.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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