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BRIEF COMMUNICATION

Cadmium induces early flowering in *Arabidopsis*

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Abstract

We found that cadmium promoted flowering in *Arabidopsis* and suppressed nitric oxide accumulation in leaves. Supplementation with NO donor SNP delayed flowering, whereas application of NO scavenger cPTIO further promoted the transition from vegetative to reproductive stage under Cd stress. Semi-quantitative RT-PCR showed that Cd treatment up-regulated the expression of *CONSTANS* and *FLOWERING LOCUS T*, whereas down-regulated the expression of *FLOWERING LOCUS C*.

Additional key words: nitric oxide, *CONSTANS*, *FLOWERING LOCUS C, FLOWERING LOCUS T.*

Cadmium (Cd) is one of the most toxic heavy metals, which has a strong inhibitory effect on plant growth. High concentration of Cd can directly or indirectly inhibit a series of physiological, anatomical and morphological parameters and even cause plant death. These processes involve photosynthesis, water relations, cell elongation, nitrogen metabolism, phytochelatin synthesis, absorption and distribution of ions (Sanita di Toppi *et al*. 1999, Durand *et al*. 2010, Kummerová *et al*. 2010, Stroiński *et al*. 2010, Xu *et al*. 2010). The most general symptoms of Cd toxicity are stunted growth, chlorosis and root necrosis. In addition, Cd also negatively affects plant reproduction by inhibiting pollen germination and ovule growth, and therefore induces invalid flower and shriveled grain (Kumar *et al*. 2005). Wani *et al*. (2007) showed that Cd application delayed flowering in chickpea plants. However, the mechanism of Cd-induced alteration in flowering time has not been clearly elucidated.

The correct flowering time is critical to successful reproduction (Simpson *et al*. 2002). There have been proposed four major pathways mediating responses to environmental and endogenous signals in *Arabidopsis*. The flowering can be regulated by the photoperiod and vernalization pathways, whereas autonomous and gibberellin (GA) pathways act independently of environmental cues (Mouradov *et al*. 2002).

The previous studies showed that nitric oxide repressed flowering in *Arabidopsis* (He *et al*. 2004). Guo *et al*. (2003) found that *AtNOS1*-deficient *Arabidopsis* plants flowered earlier than wild-type plants, and He *et al*. (2004) showed that NO-overproducing plants (*nox1*) flowered later than the wild type. NO affects flowering time by reducing the amplitude of signaling derived from the circadian clock to the photoperiod pathway (Gordon *et al*. 2005). The photoperiod pathway promotes flowering in response to day-length (Searle *et al*. 2004). NO can suppress the expression of *CONSTANS* (*CO*). In photoperiod pathway, *CO* plays a central role by linking

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Abbreviations: cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SNP - sodium nitroprusside; *CO - CONSTANS*; *FT - FLOWERING LOCUS T*; *FLC - FLOWERING LOCUS C*.
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the circadian clock and flowering time control, and *CO* mRNA expression displays a characteristic circadian rhythm (Suarez-Lopez *et al*. 2001). Flowering time would be affected if changes to either the phase or amplitude of this rhythm happened. *FLOWERING LOCUS C* (*FLC*) is a floral repressor in both the vernalization and autonomous pathways. It encodes a MADS box transcriptional factor which can inhibit the expression of a series of genes required for the transition to flowering (Levy *et al*. 1998). He *et al*. (2004) found that NO promoted the accumulation of *FLC* mRNA, thereby inhibiting flowering in *Arabidopsis* plants. In this study, we examined the effect of Cd on flowering time and the involvement of NO in the process.

Seeds of *Arabidopsis thaliana* L. Heyhn ecotype Columbia (Col-0) were sown under sterile condition in Petri dishes containing half-strength Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose and 7 g dm^{-3} agar (*Sigma*, St. Louis, USA). The seeds were stratified at 4 °C for 48 h before being transferred to a growth chamber and incubated vertically at a temperature of 25 °C and 16-h photoperiod (irradiance of 45 μmol m^{-2} s^{-1}). Seven-day-old seedlings were transferred into half-strength Hoagland solution containing various concentrations of $CdCl_2$ (0, 50 μM, 100 μM) with or without NO donor sodium nitroprusside (SNP, 100 μM) or NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 2 mM). The plants were then grown under the same culture conditions. Pictures of the seedlings were digitized using a scanner (*Epson Perfection 1670*, *SEIKO Epson Corporation*, Japan) after stress treatment. The growth of the seedlings was measured using *Image J* software, *v. 1.38* (http://rsbweb.nih.gov/ij/download.html). At least six seedlings were used in each experiment. For the statistical analysis, we used Duncan's test ($P < 0.05$).

Plantlets were oven-dried at 65 - 70 °C for 3 d. Dried tissue (50 mg) was ground up and digested in 1 cm^3 of concentrated nitric acid for 2 - 3 d at room temperature. Samples were then boiled for 1 - 2 h until completely digested. After adding 4 cm^3 of *Millipore*-filtered deionized water and brief centrifugation, the Cd content of each sample was determined by inductively coupled plasma mass spectroscopy (ICP-MS). Each experiment was repeated at least three times.

NO was monitored by incubating roots with 15 μM of the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) in 20 mM HEPES-NaOH (pH 7.5). Thereafter, the roots were washed twice for 5 min with fresh buffer and viewed under a *Leica* (Germany) laser scanning confocal microscope (excitation 490 nm, emission 515 nm).

The total RNA was isolated from the *A. thaliana* seedlings using *Trizol* (*Gibco/BRL*, Rockville, USA). For semi-quantitative RT-PCR, the concentration of RNA was accurately quantified by spectrophotometric measurements; and cDNA was synthesized from DNase-treated total RNA with a reverse transcription system kit (*Promega*, Madison, USA) using oligo-dT-primers. The genes examined and the primers used to amplify those genes were as follows: *CO* (5'-AGGTTGCTTCGT GGCTGTTC-3' and 5'-CCTGGCTTCTCTGTCCATTG-3'); *FT* (5'-CAGAGTTGTTGGAGACGTTC-3' and 5'-GCATCATCACCGTTCGTTAC-3'); *FLC* (5'-TTC TCCAAACGTCGCAACGG-3' and 5'-CCATCTCAG CTTCTGCTCCC-3') (Li *et al*. 2007). Control reactions with the *ACTIN2* primers (locus number At3g18780; 5'-CCTTCGTCTTGATCTTGCGG-3' and 5'-AGCGAT GGCTGGAACAGAAC-3') were performed to ensure that equal amounts of RNA were used in each set of reactions. Cycle numbers were optimized to ensure that the amplification reaction was tested in the exponential phase.

Cd treatment inhibited seedling growth of *Arabidopsis* markedly (Table 1). With the increasing accumulation of Cd in plants, the fresh mass decreased by 51.7 % and 72.2 % under 50 and 100 μM $CdCl_2$ treatments, respectively. Cd also reduced plant height and root length significantly (data not shown).

Though Cd toxicity inhibited vegetative growth, it promoted flowering in *Arabidopsis* as measured by the days to bolting (Fig. 1). Compared with the untreated controls, the flowering time was about 2 d earlier under 50 μM $CdCl_2$ treatment and about 4 d earlier under 100 μM $CdCl_2$ treatment (Table 1). The number of rosette leaves is a direct indicator for flowering time (Koornneef *et al*. 1991). We found that Cd treatment reduced the

Fig. 1. Cadmium treatment promoted flowering in 19-d-old *Arabidopsis thaliana*. Untreated control plant (*left*) and 50 μM Cd-treated plant (*right*). *Arrow* indicates flower bud formation.

Table 1. Effects of $CdCl_2$ treatment on growth and Cd accumulation in *Arabidopsis thaliana* seedlings. Means ± SE, $n = 6$. Different letters in the same column indicate significant differences (5 % level, Duncan's multiple range test).

$CdCl_2$ [μM]	Fresh mass [mg]	Cd content [μg g^{-1}(d.m.)]	Time to bolting [d]	Number of rosette leaves
0	58.7 ± 6.1a	0.034 ± 0.01a	21.0 ± 1.5a	11.0 ± 0.5a
50	28.3 ± 5.6b	4.960 ± 0.26b	19.0 ± 1.1b	9.0 ± 0.8ab
100	13.3 ± 1.4c	10.370 ± 0.73c	16.8 ± 0.7c	8.5 ± 0.6b

number of rosette leaves, especially when seedlings were treated with 100 μM $CdCl_2$ (Table 1).

CO/FT and *FLC* as the key regulators of the flowering transition in plants have been broadly reported (Michaels *et al*. 1999, Valverde *et al*. 2004). To further investigate the roles of *CO/FT* and *FLC* in Cd-promoted flowering in *Arabidopsis*, we next examined the expression of flowering-related genes including *CO*, *FT* and *FLC*. Cd treatment markedly induced the expression of *CO* and *FT*, whereas repressed *FLC* expression. *FLC* is a negative regulator of flowering. Up-regulation of the expression of *CO* and *FT* and down-regulation of *FLC* expression by Cd treatment suggested that *CO/FT* and *FLC* were involved in Cd-mediated early flowering in *Arabidopsis*.

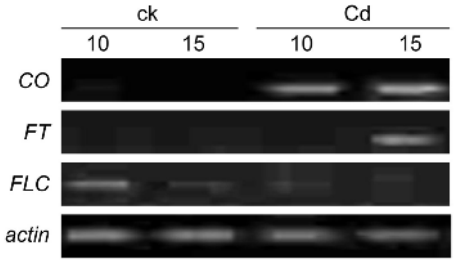

Fig. 2. Effects of 50 μM $CdCl_2$ on the expression of flowering-related genes 10 or 15 d after treatment (ck - untreated control plants).

He *et al*. (2004) showed that NO repressed flowering in *Arabidopsis*. We hypothesized that Cd-induced early flowering could be due to decreased NO production in plants. Therefore, we next examined the NO content in rosette leaves of *Arabidopsis* exposed to Cd stress. As expected, Cd repressed NO production in leaves of *Arabidopsis* (Fig. 3*A*). We found that supplementation with NO donor SNP delayed the flowering time. In contrast, application of NO scavenger cPTIO further promoted flowering under 50 μM Cd stress (Fig. 3*B*). These results indicated that NO content in leaves was involved in Cd-promoted flowering in *Arabidopsis*.

Li *et al*. (2007) found that salt stress delayed flowering in *Arabidopsis*, and *CO*-mediated floral pathway played a critical role in salt-mediated late flowering. Our study indicated that *CO*-mediated floral pathway was also involved in Cd-induced early flowering. Cd stress induced the expression of *CO* and *FT*, whereas repressed *FLC* expression, indicating that Cd promoted flowering by decreasing duration of the vegetative phase and facilitating the transition from vegetative growth to reproductive growth.

NO is a pivotal signal molecule in plants (He *et al*. 2004). NO accumulation is tightly regulated by abiotic and biotic stresses (Xu *et al*. 2009). Both Cd-regulated induction (Bartha *et al*. 2005, Besson-Bard *et al*. 2009, Mahmood *et al*. 2009) and suppression (Rodriguez-

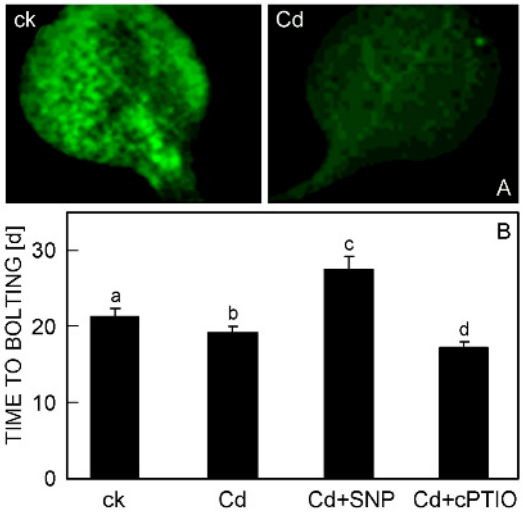

Fig. 3. *A* - Nitric oxide production in the rosette leaves of *Arabidopsis thaliana* exposed to 50 μM $CdCl_2$ for 15 d. *B* - time to bolting of plants exposed to 50 μM $CdCl_2$ with or without NO donor SNP (100 μM) or NO scavenger cPTIO (2 mM). Means ± SE, n = 6. Different letters indicate significant differences at 5 % level, Duncan's multiple range test (ck - untreated control plants).

Barroso *et al*. 2006, Serrano *et al*. 2006, Xiong *et al*. 2009) of NO accumulation in plants have been reported. The divergence might be caused by the difference in monitoring NO production. In our study, NO accumulation was observed after 15 d instead of at the early stage of Cd treatment, and endogenous NO might be induced within hours and then decreased significantly in Cd-stressed seedlings. Our study indicated that supplementation with NO donor SNP delayed flowering, whereas, reduction of NO accumulation in plants promoted flowering. We found that Cd treatment repressed NO production in plants; supplementation with NO reversed the effect of Cd on flowering. Therefore, an outline of molecular mechanism of Cd-induced early flowering was presented. Cd inhibited NO accumulation in rosette leaves. Decreased NO production in leaves led to the up-regulated expression of *CO* and *FT*, whereas to repressed expression of *FLC*, therefore, promoted flowering. Our study indicated that Cd toxicity not only inhibited vegetative growth, but also modulated reproductive growth in plants. The study is useful to further elucidate the effects of Cd stress on plant growth and provides insights into novel strategies for phytoremediation.

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