Cytokinins can act as suppressors of nitric oxide in *Arabidopsis*

Wei-Zhong Liu[a,b], Dong-Dong Kong[c], Xue-Xin Gu[d], Hong-Bo Gao[d], Jin-Zheng Wang[a], Min Xia[e], Qian Gao[a], Li-Li Tian[a], Zhang-Hong Xu[a], Fang Bao[a], Yong Hu[a], Neng-Sheng Ye[d], Zhen-Ming Pei[c], and Yi-Kun He[a,1]

[a]College of Life Sciences and [d]Department of Chemistry, Capital Normal University, Beijing 100048, China; [b]College of Life Sciences, Shanxi Normal University, Shanxi Linfen 041004, China; [c]Department of Biology, Duke University, Durham, NC 27708; and [e]Beijing Center for Physical and Chemical Analysis, Beijing 100089, China

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Maintaining nitric oxide (NO) homeostasis is essential for normal plant physiological processes. However, very little is known about the mechanisms of NO modulation in plants. Here, we report a unique mechanism for the catabolism of NO based on the reaction with the plant hormone cytokinin. We screened for NO-insensitive mutants in *Arabidopsis* and isolated two allelic lines, *cnu1-1* and *1–2* (*continuous NO-unstressed 1*), that were identified as the previously reported *altered meristem program 1* (*amp1*) and as having elevated levels of cytokinins. A double mutant of *cnu1-2* and *nitric oxide overexpression 1* (*nox1*) reduced the severity of the phenotypes ascribed to excess NO levels as did treating the *nox1* line with *trans*-zeatin, the predominant form of cytokinin in *Arabidopsis*. We further showed that peroxinitrite, an active NO derivative, can react with zeatin in vitro, which together with the results in vivo suggests that cytokinins suppress the action of NO most likely through direct interaction between them, leading to the reduction of endogenous NO levels. These results provide insights into NO signaling and regulation of its bioactivity in plants.

flowering | nitration | cross-talk | phytohormone

Nitric oxide (NO) is one of the most widespread signaling molecules in living organisms (1, 2). In plants, NO is involved in the regulation of numerous physiological processes during growth and development and is also an important modulator of disease resistance (2–4). Several laboratories discovered that NO is produced not only from nitrate/nitrite but also from L-arginine (L-Arg), which is the main substrate for NO synthesis in animals (4–6). NO is also a widespread atmospheric pollutant. Therefore, this gas not only is a pivotal player in signal transduction but also has the potential to exert significant deleterious effects by being a pollutant. As an inevitable result, increased NO levels in the atmosphere can influence multiple NO-regulated processes in organisms. Despite the wealth of information gathered from analyses of NO functioning in plants, the molecular processes underlying NO effects in plants are still largely unknown.

NO differs from other signaling molecules by being reactive, lipophilic, and volatile. In fact, chemically, NO is a free radical, and such a reactive molecule is unlikely to interact specifically with a single specific receptor (3). In animals, NO appears to act through the chemical modification of targets. NO can bind to transition metals of metalloproteins (metal nitrosylation). It also can bind covalently to cysteine (*S*-nitrosylation) and tyrosine (tyrosine nitration) residues (3, 7, 8). Such specific protein modifications are emerging as key mechanistic intermediates for NO signal transduction. In plant cells, NO has also been found to regulate the activity of various target proteins through *S*- or metal-nitrosylation and probably through tyrosine nitration as well (9–13).

Furthermore, it has been shown that NO takes part in different phytohormone signaling pathways, frequently under the control of hormonal stimuli. For instance, NO functions in auxin-induced signaling pathways driving root growth and developmental processes, operating downstream of auxin through a linear signaling pathway (14). NO acts downstream of abscisic acid in controlling both induction of stomatal closure and inhibition of stomatal opening (6, 15, 16). In addition, NO is also involved in ethylene, gibberellin, salicylic acid, and jasmonic acid signaling (17–20).

Although several lines of evidence point to the involvement of NO in cytokinin signaling (21–24), no study has yet provided definitive proof of a role for NO in cytokinin signaling (25). To date, evidence for an interaction between NO and cytokinin remains rudimentary. In this study, NO-insensitive/hyposensitive screens for *continuous NO-unstressed* (*cnu*) mutants in *Arabidopsis* were performed, and we found that *cnu1* was allelic to *altered meristem program1* (*amp1*), a known mutant having high levels of the plant hormone cytokinin. Further studies show that high levels of cytokinin suppress the action of NO most likely through direct biochemical reactions, leading to the reduction of endogenous NO levels. These findings also imply that cytokinins have protective effects against nitrosative stress by acting as NO scavengers. We showed a unique mechanism of hormone–hormone interaction in plants by which two biomolecules combine to form a new product via a direct chemical reaction.

Results

***cnu1* Mutant Is Less Sensitive to NO and Flowers Earlier Than WT.** Treating *Arabidopsis thaliana* seedlings with an NO donor, sodium nitroprusside (SNP), promotes vegetative growth at low concentrations but inhibits growth at high concentrations (4). At 120 μM SNP, vegetative growth is strongly but not completely inhibited. We used this concentration in a screen for mutants with altered NO sensitivity to identify unique components in the NO response pathway. Two independent SNP-insensitive lines were isolated that proved to be allelic based on their failure to complement SNP insensitivity in the F_1 progeny. We named these lines *cnu* (Fig. 1*A*; Fig. S1). The *cnu1-1* mutant was recovered from T-DNA insertional mutagenesis, whereas *cnu1-2* was recovered from Ethyl methane sulfonate (EMS) mutagenesis. The phenotypes of the two lines were essentially indistinguishable, and unless indicated specifically, *cnu1* refers to *cnu1-1*. Further genetic analysis indicated that *cnu1* harbors a single, recessive, nuclear mutation (Fig. S2*C*).

The insensitivity of *cnu1* plants to 120 μM SNP, as used for screening, was remarkable. When seedlings were weighed after 3 wk, the dose–response curve confirmed that *cnu1* was less responsive to SNP (Fig. 1*B*). Although the trend to less growth promotion at low SNP concentrations was not significant, the

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[1]To whom correspondence should be addressed. E-mail: yhe@mail.cnu.edu.cn.

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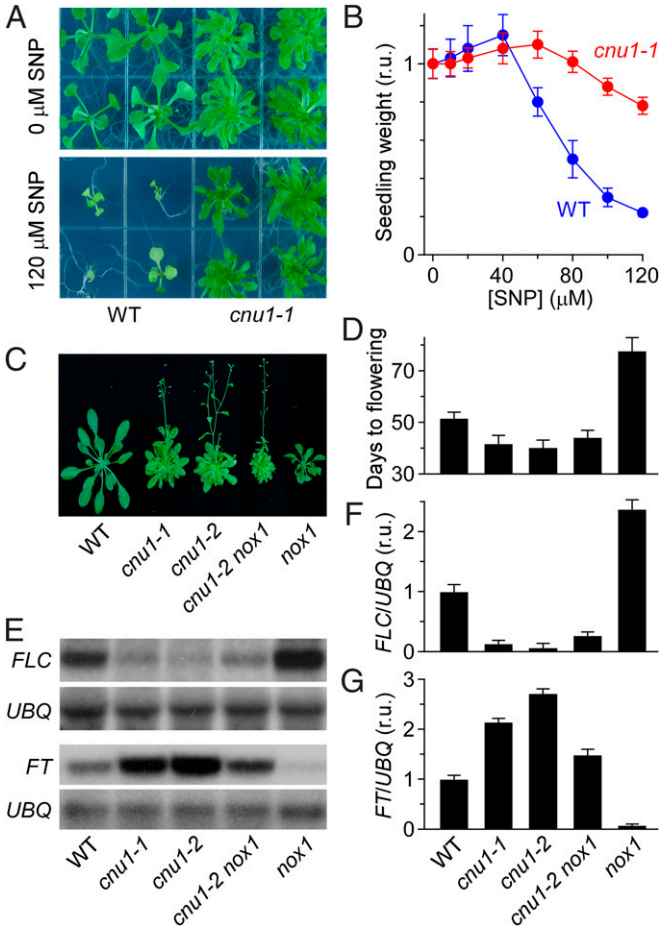

Fig. 1. The *cnu1* mutant is insensitive to NO and flowers early. (*A*) Effects of an NO donor SNP on plant growth and development. *Arabidopsis* seedlings were grown on petri dishes containing several concentrations of SNP during long days (16-h light/8-h dark) for 3 wk. It can be seen that *cnu1* mutants have started flowering, and WT plants are still in the vegetative stage at 120 μM SNP. (*B*) The effect of SNP concentration on shoot growth. Fresh weight per seedling was from experiments as in *A* (mean ± SD; n = 150 seedlings). (*C* and *D*) The variation of endogenous NO and cytokinin levels affect the onset of flowering in *Arabidopsis*. The *cnu1* mutant and *cnu1-2 nox1* double mutant flowers early. Plants were grown on soil under 12-h light/12-h dark cycles and were photographed (*C*) after 50 d of growth. The days to flowering (*D*) from experiments as in *C* were scored (mean ± SD; $n \geq 30$ plants). (*E–G*) The variation of endogenous NO and cytokinin levels affect the expression of genes that control the floral transition in *Arabidopsis*. The expression levels of *FLC* and *FT*, respectively, in WT, *cnu1-1*, *cnu1-2*, *cnu1-2 nox1*, and *nox1* plants (*E*). Seedlings were grown on MS media under 16-h light/8-h dark cycles for 10–12 d. Leaves were collected 8 h after dawn for total RNA extraction. The *FLC* mRNA abundance was analyzed by using Northern blot and *FT* mRNA by RT-PCR. Ubiquitin mRNA (*UBQ10*) was used as a loading control. Quantification of the effects of endogenous NO and cytokinin on flowering repressor *FLC* (*F*) and flowering promoter *FT* (*G*) expression, respectively, showed that endogenous NO and cytokinins on flowering control gene expression have the opposite effect. The relative mRNA abundance was normalized to the *UBQ* levels. The relative mRNA abundance of WT was arbitrarily set to 1 (mean ± SEM; n = 3).

weaker growth inhibition seen in *cnu1* at high concentrations of SNP was highly significant. For example, the ratio of seedling weight (*cnu1*/WT) increased as the SNP concentration increased (Fig. S1*A*). Additionally, inhibition of growth in *cnu1* occurred at a higher concentration (80 μM SNP) than in the WT (50 μM). The *cnu1* mutant was therefore less sensitive and less responsive to the NO donor.

Moreover, whether planted in the soil or grown under SNP treatment, *cnu1* flowered substantially earlier than WT. Quantitative analyses of flowering time showed that *cnu1* indeed flowered earlier under long day conditions (Fig. 1 *C* and *D*). Corresponding to these, RNA blot analysis showed that *FLOWERING LOCUS C* (*FLC*) expression was all but eliminated, and the *FLOWERING TIME* (*FT*) expression was increased in *cnu1* (Fig. 1 *E–G*). Consistent with previous results, growing WT plants in the presence of SNP delayed flowering (4). This response was strongly suppressed in *cnu1*, both in terms of the days to flowering and in the up-regulation of *FLC* and the down-regulation of *FT* (Fig. S1 *B–F*). Together, these results showed that both seedling growth and flowering time in the *cnu1* mutant are insensitive to NO.

***CNU1* Is Identical to *AMP1*.** The *CNU1* gene was cloned using a conventional positional cloning approach (Fig. S2*A*). Map-based cloning identified *CNU1* as At3g54720, a gene previously characterized as *AMP1* (26, 27). Sequencing analysis of the AMP1 genomic region from *cnu1* revealed a G to A point mutation in *cnu1-2*, which is identical to the mutation in *amp1-1*. PCR analysis showed that the expected product from the *AMP1* locus was detected in *cnu1-2* and in WT, but not in *cnu1-1*, which harbors a T-DNA insertion between primers locating at each end of *AMP1* gene (Fig. S2*B*). When *cnu1-1* was crossed with *amp1-1*, none of the 214 F_1 plants analyzed exhibited the WT phenotype, indicating a failure to complement (Fig. S2*C*). The morphological phenotype of *cnu1* closely resembled that described for *amp1*, including polycotyly, faster rate of leaf initiation, and early flowering time (26, 27). Finally, the *amp1* mutant responded poorly to SNP to the same extent as *cnu1* did (Fig. S2*D*). Taken together, these results corroborated the identification of *CNU1* as *AMP1*.

AMP1 encodes a putative glutamate carboxypeptidase, but its biological role remains unclear (26). In *amp1*, most likely, the enlarged meristem leads to an increase in the rate of cytokinin biosynthesis by some unknown mechanism. Thus far, several *amp1* allelic mutants (*amp1-1*, *pt/hpt*, and *cop2*, etc.) have been isolated, and all show properties associated with an increased level of cytokinin biosynthesis. The increased cytokinin in these mutants is mainly zeatin class, which increased by about sevenfold (26–29). We found that the *cnu1* mutant behaved similarly.

Zeatin Rescues the *nox1* Phenotypes Resulting from High Levels of Endogenous NO. Is *cnu1* less responsive to NO because of its elevated endogenous cytokinin levels? If so, then exogenous zeatin should rescue the delayed flowering phenotype of *nox1*, which results from the increased endogenous NO level (4). In fact, low concentrations (i.e., 0.001–1 μM) of zeatin promoted *nox1* flowering significantly (Fig. 2 *A–C*). Consistently, the expression of flowering repressor *FLC* was reduced and the expression of flowering promoter *FT* was increased in a dose-dependent manner in response to added zeatin in *nox1* (Fig. 2, *D* and *E*). Opposite responses were elicited in WT (Fig. 2), consistent with earlier reports (30). Indeed, we found that only 1 nM zeatin showed a slight effect of promoting flowering in WT. With zeatin levels >10 nM, the responses of *nox1* and WT were generally parallel, but with a weaker response for *nox1* at any given zeatin dose. Amelioration of the *nox1* delayed flowering phenotypes by zeatin is consistent with the idea that elevated cytokinin levels suppress responsiveness to NO.

Zeatin was also able to rescue the seedling growth phenotype of *nox1* (Fig. S3 *A* and *B*). Three-week-old *nox1* seedlings were about one-half the weight of WT seedlings, whereas *nox1* plants grown on 1 or 10 μM zeatin had about the same weight as untreated WT plants. This result is notable because the cytokinin decreased growth in WT. Almost 1,000 times more zeatin is required to suppress the vegetative growth of *nox1* than that of WT. These results are again consistent with a meaningful interaction between cytokinin and NO. In addition, reticulate leaf

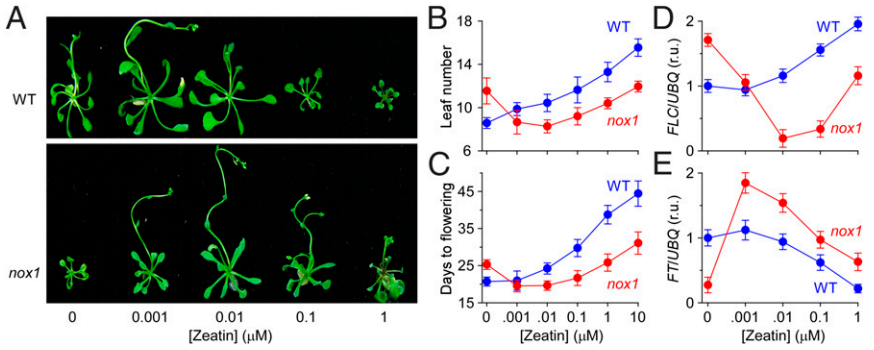

Fig. 2. Exogenous zeatin rescues the late-flowering phenotype in *nox1* resulting from elevated NO. (*A*) The cytokinin zeatin rescues the late-flowering phenotype in *nox1*. WT and *nox1* seedlings were grown on MS media containing several concentrations of *trans*-zeatin under 16-h light/8-h dark cycles and were photographed after 25 d of growth. (*B* and *C*) Quantification of flowering time measured as the days to flowering (*C*) and the number of rosette leaves (*B*) (mean ± SD; $n = 180$ seedlings) from plants grown as in *A*. (*D* and *E*) Quantification of the *FLC* (*D*) and the *FT* (*E*) expression in response to zeatin treatments, respectively, using the methods as described in Fig. 1. Seedlings were grown on media containing several concentrations of *trans*-zeatin under long days for 10–12 d. The relative mRNA abundance was normalized to the *UBQ* levels. The relative mRNA abundance of WT at 0 μM zeatin was arbitrarily set to 1 (mean ± SEM; $n = 3$).

is one of the typical phenotypes seen in *nox1* (4) and *cue1* (31) mutants. Treatment with a high concentration of SNP (120 μM) makes reticulate leaves appear in WT and also leads to a decreased chlorophyll content (4). Interestingly, when *nox1* was treated with zeatin, its reticular veins gradually disappeared as the zeatin concentration increased, traits tending to be more WT-like (Fig. S3*C*). When the zeatin concentration reached 1 μM and above, reticular veins could no longer be seen in *nox1*. Consistent with this, *nox1* chlorophyll content also increased as the concentration of zeatin increased (Fig. S3*D*).

Together, it appears that exogenous zeatin can rescue almost all of the phenotypes of *nox1* caused by elevated levels of endogenous NO in a dose-dependent manner. It is likely that cytokinin antagonizes NO either through functional antagonism or by reducing endogenous NO levels.

***cnu1 nox1* Double Mutant Has a Low Endogenous Level of NO and Flowers Early.** To further explore the relationship between elevated cytokinin levels and diminished responsiveness to NO observed in *cnu1*, we quantified NO levels using the NO-sensitive dye, 4,5-diaminofluorescein diacetate (DAF-2DA) (Fig. 3 *A* and *B*). As previously reported (4), the NO level in *nox1* was nearly 10 times higher than that of WT. Interestingly, it was found that levels of NO in *cnu1-1* and *cnu1-2* were lower than that of WT, and the NO level in the *cnu1 nox1* double mutant was only slightly elevated compared with that of WT. Compared with *nox1*, the content of NO in the *cnu1-2 nox1* double mutant decreased significantly: about 30% that in *nox1*. These results demonstrate that elevated levels of cytokinin exert a prominent negative effect on the levels of NO, an effect that might explain the diminished responsiveness to NO in *cnu1/amp1*.

In addition, the *cnu1-2 nox1* double mutants flowered much early and had lower expression of *FLC* and higher expression of *FT* than *nox1* (Fig. 1 *C*–*G*), implying that CNU1 acts downstream of the production of NO.

These results strongly suggested that the elevated levels of endogenous cytokinins made the content of NO decrease by an unknown mechanism, thus alleviating the inhibitory effect of NO in *cnu1-2 nox1*. It is possible that cytokinin alters NO levels directly.

Peroxynitrite and Zeatin React Chemically In Vitro. In cells, NO is short lived because it reacts rapidly/readily with the free radical, superoxide (O_2^-), to form the reactive molecule peroxynitrite ($ONOO^-$), a powerful oxidant that mediates numerous cellular injuries (7, 32). Compared with NO, $ONOO^-$ is longer lived, but it does react with a variety of molecules, including adenine, guanine, and xanthine nucleosides (33, 34). Natural cytokinins are all adenine derivatives and have the molecular structure needed to react directly with $ONOO^-$.

We first studied the reaction of zeatin and $ONOO^-$ in vitro. An equimolar (0.2 mM) mixture of *trans*-zeatin and $ONOO^-$ was combined at a range of pH from 4.3 to 10.5. The resulting

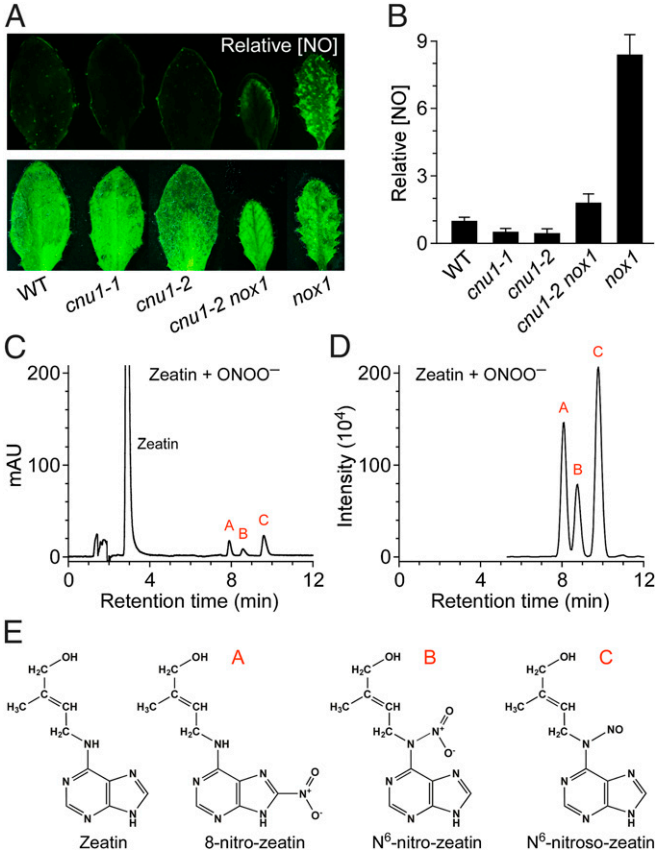

Fig. 3. Excessive NO nitrates the adenine group of cytokinins and leads to the reduction of endogenous NO levels. (*A*) The endogenous NO levels in WT, *cnu1-1*, *cnu1-2*, *cnu1-2 nox1*, and *nox1*. Leaves were stained with DAF-2DA (*Upper*). (*Lower*) White-light images are shown. A total of 50 leaves were analyzed for each genotype in three independent experiments, and similar results were obtained. (*B*) Quantification of the relative NO levels for various genotypes grown in *A*. The relative NO level of WT was arbitrarily set to 1 (mean ± SD; $n = 50$). (*C* and *D*) HPLC-UV and HPLC-MS/MS profile of the reaction products of peroxynitrite with *trans*-zeatin in test tube at pH 9.5. The reaction solution of *trans*-zeatin and peroxynitrite (Zeatin + $ONOO^-$) was run through HPLC and monitored by UV absorption (*C*) or MS/MS detection (*D*). Similar results were seen from more than 10 independent experiments. (*E*) The chemical structures of zeatin and the identified reaction products. Peaks A, B, and C in HPLC-UV (*C*) and HPLC-MS/MS (*D*) profile correspond to 8-nitro-*trans*-zeatin, N^6-nitro-*trans*-zeatin, and N^6-nitroso-*trans*-zeatin, respectively.

products were then analyzed by reverse-phase (RP)-HPLC with detection using either UV absorption or mass spectrometry. In addition to unreacted zeatin, three peaks were detected (Fig. 3 *C* and *D*). The source of peroxynitrite, synthesized either from nitrite and H_2O_2 (35) or from isoamyl nitrite and H_2O_2 (36), did not affect the formation of these peaks. However, the peaks were absent when *trans*-zeatin was mixed with previously decomposed peroxynitrite or when zeatin was mixed with sodium nitrite and H_2O_2 either alone or in combination. Similar results were obtained over a pH range from 4 to 12.

The products that were formed by a reaction of zeatin with peroxynitrite were designated as A, B, and C according to the order of elution. At neutral pH, products A, B, and C had absorbance maxima at 360, 280, and 300 nm, respectively. Product A, but not products B and C, underwent a hypsochromic shift with decreasing pH and a bathchromic shift with increasing pH (Fig. S4), which has been reported to be a characteristic of 8-nitroguanine and 8-nitroxanthine (33, 35). This observation implies nitrate addition on the purine ring of product A. The three products were studied further by collision-induced dissociation of the protonated molecular ions, which showed the same *m/z* 265 $(M+H)^+$, 529 $(2M+H)^+$ for peak A and peak B and *m/z* 249 $(M+H)^+$, 497 $(2M+H)^+$ for peak C (Figs. S5–S7). From this, the molecular formulas of A and B were determined to be $C_{10}H_{12}O_3N_6$, and of C to be $C_{10}H_{12}O_2N_6$.

The structures of the three products were further characterized by ^1H and ^{13}C NMR, including 2D NMR ^1H-^1H Correlation Spectroscopy (COSY) and Heteronuclear Multiple Quantum Correlation (HMQC) experiments, mainly on the basis of a comparison with zeatin (Table S1). The ^{13}C NMR spectrum of A showed pronounced downfield shifts in the resonances of two of the carbons: C-5 and C-8. The C-8 shift was 20 ppm downfield, consistent with enhanced electron withdrawal. In a long-range effect, the shift of C-5 in 8-nitroxanthine was about 4 ppm downfield, along with the disappearance of the C8-H around 8.1 ppm, indicating that the C8-H of zeatin was substituted with a nitro group. Furthermore, the absence of carbon-proton correlation of C8 and C8-H supported the substitution position of a nitro group at C8. The ^1H NMR spectrum of B and C showed 0.8 ppm downfield shifts in the resonances of the same H10, along with the downfield shifts of the C10 of 12 ppm for B and 1 ppm for C, which indicate that the N^6-H of zeatin was substituted with a nitro group (B) or a nitroso group (C) (Table S1). The HMQC spectra of B and C supported the substitution position at N^6.

In conclusion, we identified compound A as 8-nitro-*trans*-zeatin, compound B as N^6-nitro-*trans*-zeatin, and compound C as N^6-nitroso-*trans*-zeatin (Fig. 3*E*). In addition, we also detected the formation of three reaction products at low (50 μM) concentrations of *trans*-zeatin and $ONOO^-$ and found that the $ONOO^-$ concentrations were the yield-limiting factor of this reaction (Fig. S8 *A–D*). Similarly, N^6-isopentenyl adenine (iP), a kind of cytokinin, could also react chemically with peroxynitrite in vitro and form three products: 8-nitro-isopentenyl adenine, N^6-nitro-isopentenyl adenine, and N^6-nitroso-isopentenyl adenine, respectively.

NO and Cytokinin Interact Directly In Vivo. We determined whether a direct interaction between $ONOO^-$ and zeatin exists in vivo. An extract from *Arabidopsis* seedlings treated with 100 μM SNP was analyzed by HPLC-MS/MS (Fig. 4 *A* and *B*). A peak with a retention time matching product A was found in SNP-treated *cnu1* and WT, which gave an identical mass spectrum as 8-nitro-*trans*-zeatin, confirming that $ONOO^-$ can react with zeatin in vivo. Noteworthy, the abundance of this compound was much higher in SNP-treated *cnu1*. These results support our hypothesis that the weakened effectiveness of NO in *cnu1*/*amp1* is caused by a direct reaction between cytokinin and $ONOO^-$. This reaction leads to the reduction of endogenous NO level. Only when the endogenous cytokinin level was inadequate to react with NO, was the inhibitory effect of NO exhibited in *cnu1*. Note that, considering the effect of pH on the *trans*-zeatin and $ONOO^-$ reaction products in vitro (Fig. S8*E*), theoretically, products B and C cannot be detected in the neutral to slightly acidic environment found in the plant cytosol. Clearly, 8-nitro-*trans*-zeatin is not the only product resulted from the SNP treatment, considering there was a peak between the retention time 9–10 min in the *cnu1* mutant that was absent in WT (Fig. 4*A*).

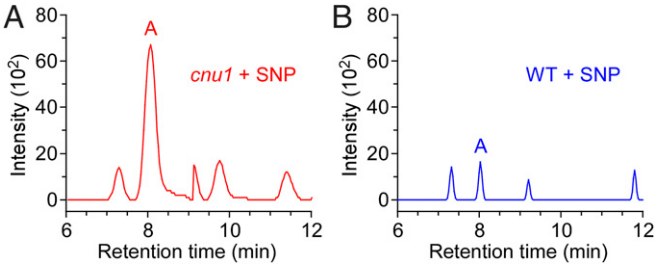

Fig. 4. Peroxynitrite and cytokinin can interact directly in vivo. HPLC-MS/MS profile of the extract from the *cnu1* (*A*) and WT (*B*) seedlings treated with 100 μM SNP. *Arabidopsis* seedlings were grown on petri dishes containing 100 μM SNP for 2 wk. Peak A corresponds to the main product A (8-nitro-*trans*-zeatin). The intensity of peak A from SNP-treated WT (WT + SNP) was much lower than that from SNP-treated *cnu1* (*cnu1* + SNP).

Cytokinin-Like Activity of the Derivatized Zeatins. Our explanation for the diminished responsiveness of *cnu1* to NO is that cytokinins essentially act like a sponge, catabolizing peroxinitrite and thereby decreasing its ambient level within the cell. However, the derivatization of zeatin will also reduce the levels of cytokinin, and therefore, the phenotype of *cnu1*/*amp1* treated with NO might also depend on the lowering of cytokinin levels. To test this, we assayed the effects of the three derivatized zeatins on root elongation and meristem cell number, both processes known to be inhibited by cytokinins (30, 37). Interestingly, although the N^6-nitro- and the N^6-nitroso-zeatin were approximately as effective as zeatin itself, the 8-nitro-zeatin was less effective. At 0.01 μM, 8-nitro-*trans*-zeatin showed almost no inhibitory effect (Fig. 5). Insofar as 8-nitro-*trans*-zeatin is predicted to be the dominant product in the acidic to neutral plant cell environment (Fig. S8*E*) and was in fact the major product detected (Fig. 4), these results suggest that treatment of plants with NO might bring about a net decrease in cytokinin activity and also suggest that the formation of 8-nitro-*trans*-zeatin can contribute to alleviating the inhibition of growth and development by NO in *Arabidopsis*.

Discussion

The interactions between NO and other phytohormones are critical to plant growth, development, and environmental responsiveness. In general, hormone–hormone interactions occur at a biosynthetic level, a signaling level, or via a common action on a specific process (38). Herein, we integrated biological and chemical analysis to show that NO can interact directly with cytokinins, most likely through NO nitrating cytokinins, further modulating each other's homeostatic levels and bioactivity. This interaction is a unique mechanism of NO–hormone interaction in plants by which two bioactive molecules combine to form new products via a chemical reaction.

During the last few years, the accumulated knowledge on NO and cytokinin physiological effects and response pathways have been adding pieces to the puzzle of the relationship between NO and cytokinin. The synergistic effects of NO and cytokinin in the regulation of photomorphogenesis (2), growth (4), and senescence (2) as opposed to their antagonistic effects in the control

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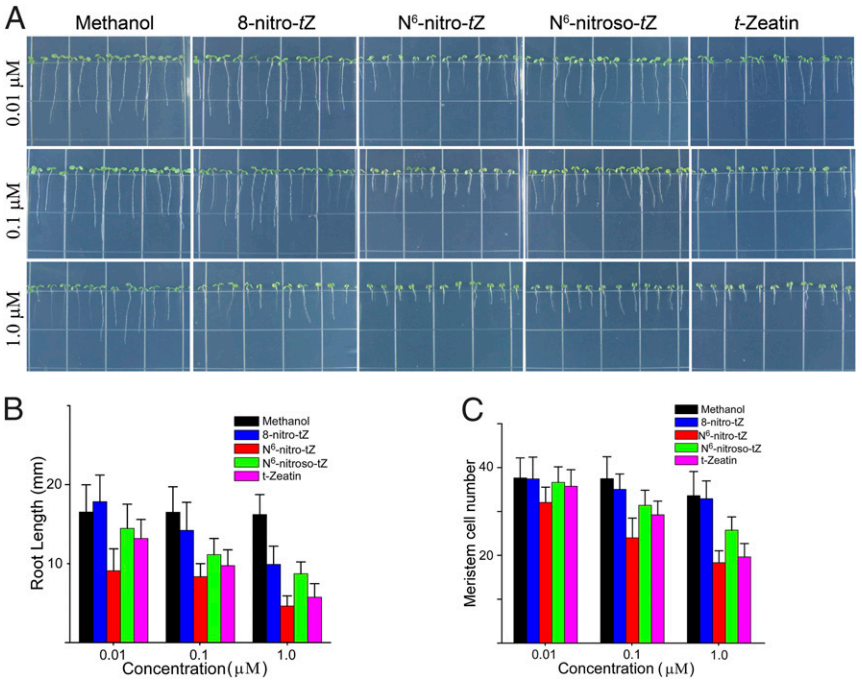

Fig. 5. Cytokinin-like activity of products A, B, and C. (*A*) Root phenotypes of seedlings grown under long days for 1 wk on the indicated treatments. WT seeds were grown on MS media each containing several concentrations of 8-nitro-*trans*-zeatin, N^6-nitro-*trans*-zeatin, N^6-nitroso-*trans*-zeatin, *trans*-zeatin, and methanol, respectively. All compounds were dissolved in methanol, which is shown at the same concentration as a control. (*B*) The root length from experiments as in *A* was analyzed with Image J software (mean ± SD; n = 160 seedlings). (*C*) Effects of these three products on the root-meristem cell number. Root-meristem size was expressed as the number of cortex cells in a file extending from the quiescent center (QC) to the first elongated cell. The meristem sizes of roots treated with these three products were analyzed using the number of cortex cells (mean ± SD; n = 160 seedlings).

of seed germination (30, 39), flowering (4, 40, 41), and stomatal closure (42, 43) in *Arabidopsis* illustrate some of the complexities of the NO–cytokinin interaction. Multilevel or multitype interactions may be involved in the cross-talk between NO and cytokinin. Previously, it was reported that the exogenous application of cytokinins to tobacco, parsley, maize, and *Arabidopsis* cell cultures leads to a rapid stimulation of NO release, suggesting a positive correlation between cytokinin and NO production (22–24). However, conversely, our data show that elevated cytokinin endogenously not only failed to induce NO production but also decreased the content of NO in vivo (Fig. 3 *A* and *B*). In addition, Wilhelmova et al. (44) also found a negative correlation between cytokinin levels and NO production using transgenic tobacco plants with genetically increased or decreased levels of cytokinins. Clearly, the observations from the experiments applying exogenous NO or cytokinins are inconsistent with the results obtained using mutants or transgenic plants with altered NO or cytokinin levels.

Exogenously applied NO or cytokinin may not faithfully replicate the function of endogenous NO or cytokinin and may have side effects in plants. Furthermore, the application of a high concentration of cytokinins may result in stresses (inhibiting/damaging effects), and most all abiotic and biotic stresses induce the production of NO (3). Therefore, it is difficult to determine whether the production of NO is induced by cytokinin itself or by a cytokinin-induced stresses. The reported concentrations of cytokinins that can induce NO biosynthesis have been shown to be rather high and themselves might cause cell damage, whereas a highly effective (but not damaging) cytokinin concentration [4 μM 6-benzylaminopurine (BA)] did not induce NO release from suspension-cultured *Arabidopsis* cells (22). Further evidence from the work of She and Song (43) showed that added cytokinins [≤0.6 μM 6-BA or Kinetin (KT)] not only reduced NO levels in guard cells caused by SNP treatment in the light but also eliminated NO that had been generated by dark, and then promoted the closed stomata reopening, just as was seen with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Thus, the genetic-based approach, i.e., analysis of mutants with altered endogenous NO or cytokinin levels, is likely to be more accurate for dissecting the nature of the interactions between NO and cytokinin in *Arabidopsis*.

Even more importantly, while dissecting the nature of interaction between NO and cytokinin, we should also give special attention to the unique chemical properties of NO and its derivatives, especially their strong oxidant activities.

In this study, by dissecting the effects of high levels of cytokinins on the NO insensitivity in *cnu1*, we provided several lines of evidence to demonstrate that cytokinins down-regulate endogenous NO levels by reacting directly with $ONOO^-$ in *Arabidopsis*, which also explains the underlying mechanism of the NO insensitivity in the *cnu1* mutant. First, we observed that exogenously applied zeatin rescued almost all of the phenotypes of *nox1*, including the delayed flowering phenotype, caused by elevated levels of endogenous NO (Fig. 2; Fig. S3). Second, the *cnu1 nox1* double mutant displayed a significantly decreased level of endogenous NO and flowered early, implying that cytokinins function as a negative regulator of NO (Figs. 1 *C*–*G* and 3 *A* and *B*). Third, chemical data showed that $ONOO^-$ was able to react with *trans*-zeatin to generate three products across a pH range from 4.3 to 10.5 (Fig. 3; Figs. S5–S8*E*). In the normal plant cell environment (pH 5.5–7.5), 8-nitro-*trans*-zeatin was the dominant product and had almost no biological activity (Fig. S8*E*; Fig. 5). Finally, 8-nitro-*trans*-zeatin was detected in the tissue extract from SNP-treated *Arabidopsis* plants, and its content in SNP-treated *cnu1* was much higher than that in SNP-treated WT plants (Fig. 4). The evidence for a direct interaction between $ONOO^-$ and zeatin in vivo also exists. It is important to point out that the product formed by the reaction of zeatin with $ONOO^-$ in vivo is much less than the trace amount of zeatin; thus far, it is almost impossible to purify sufficient products from the extract for analysis, so the identity of the product can only be determined by detecting the peaks of HPLC-MS/MS. The data presented here indicate that the type of interaction between NO and cytokinins should be the effect of one of the substances on the level of another. It is achieved through chemical combination of two bimolecules, rather than cytokinin regulation of NO synthesis. However, it should be noted that the direct reaction between NO and cytokinins in plant cells might occur only in a limited range because the NO content in *cnu1-2 nox1* was not reduced completely to the WT level (Fig. 3 *A* and *B*).

The fact that NO and cytokinins can react chemically also implies that cytokinins can modulate NO homeostasis and have protective effects against reactive nitrogen species in plant cells. It has been described that NO possesses both cytotoxic and cytoprotecting/stimulating dual properties, which depends on the concentration and the local biochemical microenvironment in plant cells (45). The wide variety of sources of NO and its possible effects suggest the need for detoxification mechanisms to modulate its level and functioning. An increasing number of reports have implicated nonsymbiotic hemoglobins as the key enzymatic system for NO scavenging in plants (9). Based on the results of this study, it appears that plants possess more than one way to attenuate high NO stress. In addition to hemoglobin, cytokinins are also involved, at least to some extent, in modulation of NO levels in plants. This finding provides an efficient mechanism for a rapid removal of toxic levels of NO. The existence of a variety of NO modulation reactions indicates that specific NO detoxification mechanisms may be involved in the fine control of the level and functions of NO under specific plant conditions. Interestingly, previous studies have also reported that cytokinins can have protective effects against the damage caused by reactive oxygen species (46, 47). Accordingly, we believe that, as a biomolecule with antioxidant properties, cytokinins reacting directly with reactive nitrogen species to scavenge excessive NO is reasonable.

Taken together, we revealed a unique mechanism for NO–hormone interaction in plants whereby excess NO nitrates the adenine group of cytokinins to decrease its own endogenous level, implying that cytokinins have protective effects against nitrosative stress by scavenging NO. These findings provide insights into NO signaling and homeostasis in plants.

Materials and Methods

Plant materials and growth conditions, isolation and positional identification of *cnu* mutants, analysis of flowering time and mRNA abundance, detection of endogenous NO levels, chemical details about the purification and characterization of products formed by the reaction of *trans*-zeatin and peroxynitrite, and other procedures are fully described in *SI Materials and Methods*.

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