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Interactions between nitric oxide, gibberellic acid, and phosphorus regulate primary root growth in *Arabidopsis*

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Abstract

Nitric oxide (NO), gibberellic acid (GA), and phosphorus (P) have been reported to regulate primary root (PR) growth, but interactions between them in the growth of *Arabidopsis* PR remain unknown. This work confirmed that low P availability significantly inhibited PR growth and that NO arrested PR growth in either high P or low P conditions. Moreover, NO counteracted the stimulatory effects of GA on PR growth under low P conditions. Finally, the dependence of low P and NO inhibition of PR growth on the DELLA-SLY pathway revealed that NO stabilized a major DELLA protein. We therefore conclude that antagonistic interactions between NO and GA regulate PR growth under both the high and low P conditions, and a DELLA-SLY module is the node where NO, GA, and P pathways converge and interact.

Additional key words: cPTIO, DELLA proteins, SNP.

Introduction

Nitric oxide has been reported to facilitate photomorphogenesis (Beligni *et al.* 2000) and flowering (Zhou *et al*. 2012), and to mediate stomatal closure (García-Mata *et al*. 2001) and stress adaptations (Graziano *et al*. 2002, Peto *et al*. 2011). Recently, NO is also considered to be an important regulator of root growth and development (Fernández-Marcos *et al*. 2011). For example, NO inhibits primary root (PR) growth and stimulates lateral root (LR) formation in tomato (Correa-Aragunde *et al*. 2004, 2006). NO also stimulates adventitious rooting in cucumber cuttings (Pagnussat *et al*. 2003) and acts as the downstream player of auxin in the stimulation of root hair proliferation (Lombardo *et al*. 2006).

Gibberellic acids (GAs) are well known for promoting PR growth (Fu and Harberd 2003) and repressing shoot senescence (Yu *et al*. 2012). A collection of nuclear localized proteins, GA insensitive (GAI), repressor of *ga1-3* (RGA), RGA-like 1 (RGL1), RGL2, and RGL3, have been found to share a conserved DELLA motif and act as negative transcription factors in GA signalling (Sun and Gubler 2004). More specifically, RGA plays a dominant inhibitory role in PR growth (Fu and Harberd 2003) in *Arabidopsis* and rice, whereas F-box proteins, SLEEPY 1 (SLY1) and GA-insensitive dwarf2 (GID2), have been identified as the part of E3 ubiquitin ligase SCF (Skp1-Cullin-F-box complex), and they are involved in DELLA protein degradation (McGinnis *et al*. 2003, Sasaki *et al*. 2003). The fact that salt stress and ethylene arrest PR growth through stabilization of DELLA proteins is also noteworthy (Achard *et al*. 2003, 2006). Interestingly, phosphate (Pi) starvation decreases GA biosynthesis in *Arabidopsis* (Jiang *et al*. 2007).

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Abbreviations: cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; GA - gibberellic acid; GAI - GA-insensitive; GID2 - GA-insensitive dwarf2; L-NMMA - L-NG-monomethylarginine; LR - lateral root; NOS - nitric oxide synthase; Pi - inorganic phosphate; PR - primary root; RGL 1 - RGA-like 1; SCF - Skp1-Cullin-F-box complex; SLY 1 - SLEEPY 1; SNP - sodium nitroprusside.

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Low P availability inhibits PR growth (Williamson *et al*. 2001, López-Bucio *et al*. 2002) and plants have evolved several adaptive mechanisms to cope with Pi starvation (López-Bucio *et al*. 2002). In response to low P availability, plants may significantly remodel root system architecture through an increased LR density, an increased root hair number and length, and a shortened PR length with the aim to increase the P absorption (Williamson *et al*. 2001, López-Bucio *et al*. 2002).

Based on the overlapping roles of GA and NO in root growth and photomorphogenesis, we hypothesized that the interactions between NO and GA are also involved in the regulation of PR growth by P availability. Our hypothesis was that antagonistic interactions between NO and GA can regulate PR growth under both Pi-sufficient and Pi-deficient conditions, and that a DELLA-SLY module may be the node of convergence for the interactions between NO, GA, and P.

Materials and methods

Plants, growth, and treatments: The background for *rga, gai, rgl1, rgl2, rgl3, sly1-D,* and *rga gai* mutants and a double mutant was *Arabidopsis thaliana* L. genotype Lansberg erecta (Ler*)*. Controls were wild type Ler. Seeds were surface sterilized with 70 % (v/v) ethanol for 5 min and then washed with 10 % (m/v) NaClO for 5 min. After five rinses with sterilized distilled water, the seeds were dried on sterilized paper. The seeds were then germinated on plates with modified Murashige and Skoog (MS) media solidified with *Phytagel* (*Sigma-Aldrich*, St. Louis, USA; 5 g dm^{-3}) which contained 5 g dm^{-3} sucrose, 10 mg dm^{-3} inositol, 0.2 mg dm^{-3} glycine, and varying concentrations of KH_2PO_4 (from 5 to 500 μM). Modified media contained 10.3 mM NH_4NO_3, 9.4 mM KNO_3, 1.5 mM $CaCl_2 . 2\,H_2O$, 0.75 mM $MgSO_4 . 7\,H_2O$, 2.5 μM KI, 50 μM H_3BO_3, 0.05 mM $MnSO_4 . 1\,H_2O$,18.5 μM $ZnSO_4 . 7\,H_2O$, 0.5 μM $Na_2MoO_4 . 2\,H_2O$, 0.05 μM $CuSO_4 . 5\,H_2O$, 0.05 μM $CoCl_2 . 6\,H_2O$, 50 μM $FeSO_4 . 7\,H_2O$, and 50 μM $Na_2EDTA . 2\,H_2O$. The pH was adjusted to 5.7 with HCl or NaOH. Then the plates were wrapped with a double layer of foil and refrigerated at 4 °C for 4 d to break dormancy. Upon removal from the refrigerator, the sterilized seeds were germinated and grown for 7 d under a 16-h photoperiod with an irradiance of 100 μmol $m^{-2}\,s^{-1}$, day/night temperatures of 22/20 °C, and a 70 % relative humidity. Roots of the seedlings were scanned (*Epson 1460XL*, Nagano, Japan) at a resolution of 400 dpi. The PR length was measured by *ImageJ 1.33* (National Institutes of Health, Bethesda, USA) (Abramoff *et al*. 2004).

GA, sodium nitroprusside (SNP), 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and L-NG-monomethylarginine (L-NMMA) were all purchased from *Sigma-Aldrich* (St. Louis, USA). GA was initially dissolved in 0.2 cm^3 of ethanol, and then water was added to make 10 cm^3 of 1 mM stock solution. SNP, cPTIO, and L-NMMA were dissolved in water. Ultrafiltration-sterilized chemicals were added to the cooled molten high P or low P media in appropriate volumes to reach the final concentrations as indicated.

Detection of GFP fluorescence: A *Leica SP2* (Wetzlar, Germany) inverted laser confocal microscope was used. To detect GFP signalling, the excitation wavelength was 488 nm, and the band path filter of 510 to 525 nm was set for the emission. Root tips from the 7-d-old transgenic *Arabidopsis* plants expressing a *pRGA:GFP-RGA* construct and grown under the high or low P media containing 0 or 10 μM SNP were mounted on standard microscope slides. The slides were sealed using nail polish. The root tips were scanned using laser confocal microscopy. After obtaining the image, the average green fluorescent signals in selected whole root sections were quantified by *ImageJ 1.33*. Photographs were processed with the *GIMP 2.8* software.

Statistical analysis: For all the experiments, data were analyzed with the *SAS v. 8.1* software (*SAS*, Chicago, USA). The differences among treatments and controls were evaluated with the Duncan's multiple range test at $\alpha = 0.05$.

Results

To test the feasibility of our experimental conditions and to explore the effects of Pi availability on PR growth, *Arabidopsis* plants were grown in media containing different KH_2PO_4 concentrations ranging from 5 to 500 μM (Table 1). The Pi availability regulated PR growth in a dose-dependent manner, *e.g.*, the length of PRs at 5 μM Pi was only 40 % of that at 500 μM Pi. Therefore, 5 μM Pi was considered as Pi-deficient (LP) and 500 μM Pi was considered as Pi-sufficient (HP) in following experiments.

Next, the PR elongation rate was measured every day after germination under the LP and HP conditions, and LP significantly reduced the elongation rate of PR after the third day, and the inhibitory effect became more and more obvious over time (data not shown).

To determine effects of exogenous NO on PR growth,

Table 1. The effects of P supply on PR growth. The length of PR in HP (500 μM KH_2PO_4, control) was set as 100 %. Means ± SE, n = 3. Different letters indicate significant difference at $P < 0.05$ between the treatments based on the Duncan's multiple range test.

P concentration [μM]	PR length [% control]
5	40.0 ± 1.1c
10	41.0 ± 4.2c
25	43.0 ± 4.4c
50	59.0 ± 4.0b
100	65.0 ± 4.9b
500 (control)	100.0 ± 1.7a

Table 2. The NO inhibition of *Arabidopsis* seedling PR growth under high P (500 μM) or low P (5 μM) concentrations as determined by the treatment with a NO donor (SNP) alone or in combination with a NO scavenger (cPTIO). Means ± SE, n = 3. Different letters in the same phosphorus and cPTIO group indicate significant differences (the Duncan's multiple range test, $P < 0.05$).

P concentration [μM]	SNP [μM]	cPTIO [μM]	PR length [% control]
500 (control)	0	0	100.0 ± 4.3a
500	1	0	74.3 ± 6.9b
500	10	0	39.6 ± 6.1c
500	100	0	24.2 ± 0.6d
5(control)	0	0	100.0 ± 9.7a
5	1	0	82.1 ± 11.9ab
5	10	0	39.2 ± 2.9c
5	100	0	28.9 ± 1.7d
500 (control)	0	0	100.0 ± 4.4a
500	10	0	72.6 ± 3.8c
500	10	10	76.9 ± 1.6bc
500	10	30	83.9 ± 2.6ab
500 (control)	0	0	100.0 ± 13.0a
5	0	0	42.0 ± 4.3c
5	0	10	57.0 ± 12.0bc
5	0	30	81.0 ± 7.80b

the NO donor SNP (1 - 100 μM) was applied to the LP and HP grown seedlings for 7 d. SNP inhibited PR growth under both the HP and LP conditions, particularly in higher concentrations (10 and 100 μM; Table 2). To rule out side effects of SNP on PR growth, the seedlings were treated with a combination of SNP and cPTIO, a scavenger of NO (Pagnussat *et al*. 2003, Foresi *et al*. 2010). The addition of 30 μM cPTIO partially blocked the SNP inhibition of PR growth (Table 2) under HP. This suggests that the effects of NO on PR growth under HP might be direct. Furthermore, the exposure to 10 and 30 μM cPTIO alone partially rescued the reduction of PR growth induced by LP (Table 2). This indicates that the endogenous NO might inhibit PR growth under low P.

L-NMMA is an inhibitor of NO synthase and the application of 1 μM L-NMMA significantly counteracted the inhibitory effects of LP on PR growth, whereas 10 μM L-NMMA slightly but not significantly stimulated PR growth, and 50 μM and 100 μM L-NMMA inhibited PR growth under LP (Table 3). These results imply that the inhibition of PR growth by low P might rely on the NO synthesis pathway.

Table 3. The dependence of a low P inhibition of PR growth on endogenous NO. The length of PR in HP (control) was set as 100 %. Two independent experiments with similar results were carried out. Results are presented from one experiment. Means ± SE, $n \geq 34$. Different letters indicates significant differences of means (the Duncan's multiple range test, $P < 0.05$).

P concentration [μM]	L-NMMA [μM]	PR length [% control]
500 (control)	0	100.0 ± 19.6a
5	0	18.6 ± 3.7c
5	1	42.8 ± 4.0b
5	10	26.6 ± 2.6c
5	50	9.0 ± 0.7d
5	100	10.0 ± 0.5d

Table 4. The stimulation of PR growth in LP by GA and SNP. The length of PR in HP (control) was set as 100 %. Means ± SE, n = 3. Different letters indicate significant differences between treatments (the Duncan's multiple range test, $P < 0.05$).

P concentration [μM]	GA [μM]	SNP [μM]	PR length [% control]
500 (control)	0	0	100.0 ± 4.7a
5	0	0	37.0 ± 7.6d
5	0.1	0	48.4 ± 9.6cd
5	1	0	69.7 ± 3.6b
5	10	0	64.4 ± 7.2bc
5	20	0	50.1 ± 10.0cd
500 (control)	0	0	100.0 ± 5.9a
5	0	0	32.0 ± 1.5c
5	1	0	72.0 ± 2.1b
5	1	10	40.0 ± 2.0c

To link P and GA in the regulation of PR growth, the effects of GA on PR growth under the LP conditions were explored. As expected, the application of GA promoted PR growth (Table 4). For instance, 1 and 10 μM GA significantly stimulated PR elongation under the LP conditions, whereas 0.1 and 20 μM GA slightly but not significantly boosted PR growth (Table 4). These results indicate that GA was a positive regulator for PR growth under the LP conditions.

To explore the interactions between NO and GA in PR growth under the LP conditions, the seedlings were treated with GA and SNP simultaneously. Under the LP conditions, GA (1 and 10 μM) significantly but not completely rescued PR growth. Moreover, the application of 10 μM SNP attenuated the stimulatory effects of 1 μM GA on PR growth (Table 4). These results indicate that GA is antagonistic to NO at relevant concentrations.

Considering DELLAs are central players for PR growth and SLY1 is the major F-box protein involved in degradation of DELLAs (Dill *et al*. 2004, Fu *et al*. 2004), the involvement of the DELLA-SLY pathway in the interaction between GA and NO was therefore explored. Under the HP conditions, 10 μM SNP retarded PR growth to 30 % of that in the control wild type Ler. In contrast to the Ler, a loss of function mutation in *GAI*, *RGL1*, *RGL2,* and *RGA* attenuated the inhibitory effects of 10 μM SNP on PR elongation. Moreover, relative to a single DELLA mutation, double loss of function mutations in *RGA* and *GAI* significantly increased PR elongation. As reported, *sly1-D*, a gain of function mutant of *SLY1*, has enhanced degradation of the DELLA proteins in contrast to wild type because of the higher affinity of SLY with DELLA proteins (McGinnis *et al*. 2003). Interestingly, this gain of function mutation in *SLY1* significantly masked the inhibitory effects of 10 μM SNP on PR growth. The relative length of the PR in *sly1-D* was significantly higher than that in the *rga* mutant (Fig. 1*A*). These results strongly suggest that the inhibitory effects of SNP on PR growth under HP were dependent on the DELLA-SLY pathway.

To test whether the inhibitory effects of P starvation in PR growth depends on the DELLA-SLY pathway, related mutants were tested. Relative to the wild type, loss of function mutations in *GAI* or *RGL1* slightly but not significantly increased PR growth, whereas a loss of function mutation in *RGA* significantly promoted PR growth indicating that RGA is the major repressor for PR growth under LP. A double mutation in *GAI* and *RGA* obviously masked the inhibitory effects of P deficiency on PR growth. Moreover, a gain of function in *SLY1* nearly fully counteracted the inhibitory effects of LP on PR growth. These results also indicate that P starvation inhibited PR growth *via* the DELLA-SLY pathway.

As RGA is the major repressor for PR growth (Fig. 1*A*,*B*), and NO inhibited PR growth *via* DELLA proteins under the HP conditions (Fig. 1*A*), it is therefore conceivable that NO stabilizes DELLA proteins. To test this hypothesis, GFP-RGA signaling was observed using confocal microscopy. The RGA protein was localized in the nucleus as previously reported (Fu *et al.* 2004). The application of 10 μM SNP increased the RGA::GFP signal in both HP (Fig. 2*A*,*B*,*E*) and LP (Fig. 2*C*,*D*,*E*) indicating that exogenous NO stabilized the RGA protein in root tip nuclei. Furthermore, relative to HP (Fig. 2*A*), LP inhibited the degradation of RGA in roots (Fig. 2*A*,*D*). These results indicate that Pi availability altered the abundance of the RGA protein in roots, and NO inhibited PR growth probably through stabilizing RGA.

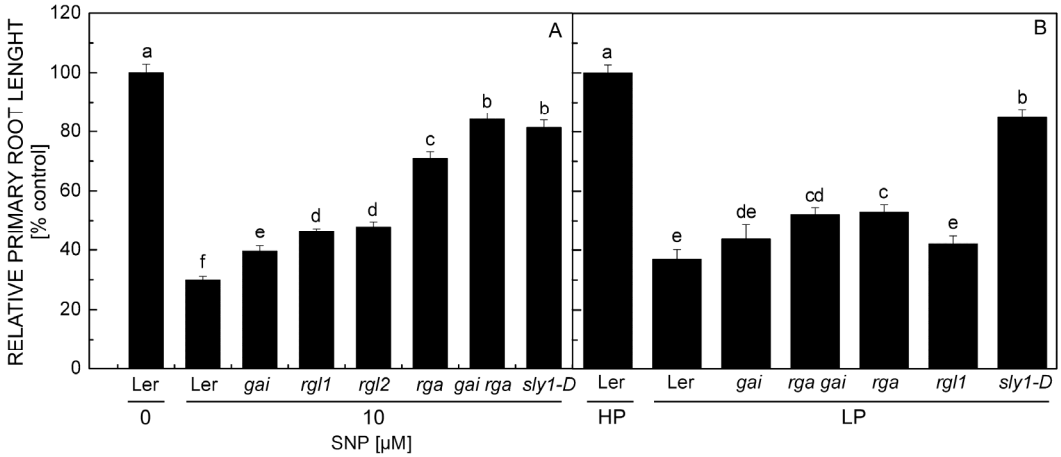

Fig. 1. The DELLA-SLY pathway-dependence of the inhibitory effect of NO in high P, and of low P on PR growth. *A* - The effects of NO on PR growth in HP. The length of PR in HP (control Ler/0) was set as 100 %. The experiments were repeated twice with similar results. Data are presented from one experiment ($n \geq 21$). Different letters indicate significant differences at the $P < 0.05$. *B* - The effects of LP on PR growth. The length of PR in HP (control; Ler/HP) was set as 100 %. The experiments were repeated twice with similar results. Data are presented from one experiment ($n \geq 21$). Different letters indicate significant differences at the $P < 0.05$.

Discussion

Nitric oxide has been documented to interact with ABA to regulate stomatal closure and drought tolerance (Desikan *et al.* 2002), as well as with auxin to modulate root growth and development (Pagnussat *et al*. 2003,

Correa-Aragunde *et al*. 2004). In this study, we found that NO interacted with GA to regulate PR growth.

It has also been reported that NO inhibits PR growth under normal nutrient conditions (Fernandez-Marcos *et al*. 2011). In line with these results, in the current study, the high content of NO inhibited PR growth in *Arabidopsis* under both the HP and LP conditions (Table 2). These results suggest that NO was involved in modulating the effects of P availability on PR growth. As expected in this model, the scavenger of NO, cPTIO, blocked the inhibitory effects of SNP on PR growth which further verified that NO directly inhibited PR growth. A likely mechanism is that low P led to increased endogenous NO production.

Recently, NO synthase from the green alga *Ostreococcus auri* was characterized, and L-NMMA was reported to decrease endogenous NO emission (Foresi *et al*. 2010). Here, we report that the addition of L-NMMA attenuated the inhibitory effects of LP on PR growth, indicating LP inhibited PR elongation *via* NOS, at least in part. Moreover, nitrate reductases 1 (NIA1) and 2 (NIA2) contribute to a large fraction of NO emissions in *Arabidopsis* (Desikan *et al*. 2002). Therefore, it would be interesting to test the roles of NIA1 and NIA2 in the P regulation of PR growth *via* the employment of *nia1* and *nia2* single or double mutants in the future. NO also plays important roles in mediating nitrate-dependent root growth in maize (Zhao *et al*. 2007). In addition, NO is the shared signalling molecule for phosphorus- and iron-deficiency induced formation of cluster roots in white lupin (Meng *et al*. 2012). The sum of this and previous studies indicate that NO is an important modulator for root system remodeling in response to a range of nutrient stresses.

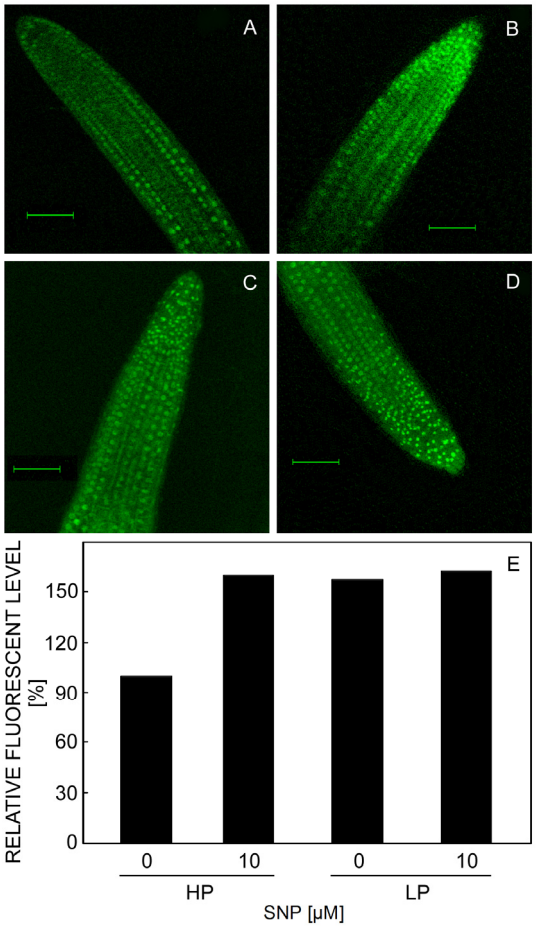

Fig. 2. NO stabilized the RGA protein in primary root tips of *Arabidopsis*. A *pRGA:GFP-RGA* line was employed to observe the RGA protein content in root tips. GFP signals in root tips of 7-d-old *Arabidopsis* seedlings in media supplied with 500 μM Pi (HP) or 5 μM Pi (LP) were detected using *Leica SP2* laser confocal microscopy: *A* - HP, *B* - HP + 10 μM SNP, *C* - LP, *D* - LP + 10 μM SNP. *E* - Relative average green fluorescence signal measured with *ImageJ 1.33* based on the whole root sections in *A*, *B*, *C*, and *D*.The green fluorescence in (*A*) was set as 100 %.

Our results clearly show that the inhibitory effect of NO and LP on PR growth depended on the DELLA-SLY pathway (Fig. 1*A*,*B*). It has been established that LP reduces the content of GA which in turn results in stabilized DELLA proteins in the root tip (Jiang *et al*. 2007). Here, it is demonstrated that under the HP conditions, loss of function mutations in DELLA proteins attenuated the inhibitory effects of NO on PR growth (Figs. 1 and 2), particularly if the mutation is in RGA (Fig. 1). This indicates that NO inhibited PR elongation *via* the DELLA pathway. As expected, a gain of function mutation in *SLY1*, an F-Box protein responsible for DELLA protein degradation, sharply masked the negative effects of NO on PR growth, but did not fully rescued PR growth. This suggests that NO inhibition of PR growth depended on the GA-DELLA-SLY pathway, but not entirely. In line with these results (Fig. 1), salt inhibits PR elongation in the quadruple DELLA mutant *gai rga rgl1 rgl2* less than in the wild type Ler (Achard *et al*. 2006). This hints DELLA proteins are central integrators of environmental effects on growth and development.

Further evidence for the modulation of PR growth by gibberellins is seen in an observation that salt-treated wild type *Arabdidopsis* contains less bioactive GA_1 and GA_4 (Achard *et al*. 2006). Pi starvation also reduces the GA content in *Arabidopsis via* down-regulation of the *GA20ox1* expression (Jiang *et al*. 2007). It is also notable that NO counteracts the GA regulation of hypocotyl growth under low radiation by decreasing the GA content *via* down-regulation of the *GA20ox3* expression (Lozano-Juste *et al*. 2011). These studies implied that both NO and P availability control GA biosynthesis through the modulation of the *GA20ox* gene expression which then affects PR growth.

In summary, our study revealed that NO arrested PR growth under both the HP and LP conditions, and counteracted stimulatory effects of GA on PR growth under the LP conditions. More importantly, the inhibitory effects of LP and NO on PR growth depended on the

GA-DELLA-SLY pathway. Finally, a DELLA-SLY module appears to be the convergence node for crosstalk between NO, GA, and phosphorus.

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