Nitro-Phenlactone, a Carlactone Analog with Pleiotropic Strigolactone Activities

Dear Editor,

Strigolactones (SLs) are novel phytohormones that shape plants architecture by inhibiting shoot branching and regulating root growth, besides their established functions in stimulating seed germination of root parasitic weeds, such as *Striga* and *Phelipanche* species, and inducing hyphal branching in arbuscular mycorrhizal (AM) fungi (Al-Babili and Bouwmeester, 2015). Canonical SLs are divided into strigol- and orobanchol-like subfamilies with a typical structure consisting of a tricyclic (ABC-ring) and a monocyclic lactone (D-ring), which are connected by an enol ether bridge (Figure 1A and Supplemental Figure 1). SLs are synthesized from carotenoids via carlactone, which lacks the B/C-ring (Alder et al., 2012). In Arabidopsis, carlactone (Figure 1A) is converted by MAX1, a member of CYP711 family, into carlactonoic acid that can, after methylation, bind to SL receptor (Abe et al., 2014). In rice, a MAX1-homolog, the carlactone oxidase, introduces B/C-rings into carlactone, yielding 4-deoxyorobanchol (Supplemental Figure 1) (Zhang et al., 2014), the precursor of canonical orobanchol-like SLs. SL perception involves an α/β-fold hydrolase (D14 in rice) that acts as a receptor and the SCF-type ubiquitin ligase MAX2 (D3 in rice) that mediates degradation of target proteins, such as members of the SMAX1-LIKE (SMXL; D53 in rice) family of chaperonin-like proteins (Bennett and Leyser, 2014). By interacting with D14-homolog KAI2, MAX2 mediates also signal transduction of karrikins, smoke-derived compounds that act as growth regulators inhibiting hypocotyl growth and inducing seed germination in Arabidopsis, but not in root parasitic weeds (Smith and Li, 2014).

Our knowledge of SL functions is largely deduced from the use of SL analogs, particularly GR24 (Figure 1A), that were developed based on the structure of canonical SLs. GR24 and other SL analogs could also be employed in agriculture to combat witchweeds, which cause enormous yield losses in several crops, by triggering parasite

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Our knowledge of SL functions is largely deduced from the use of SL analogs, particularly GR24 (Figure 1A), that were developed based on the structure of canonical SLs. GR24 and other SL analogs could also be employed in agriculture to combat witchweeds, which cause enormous yield losses in several crops, by triggering parasite
seed germination in the absence of hosts (suicidal germination) (Zwanenburg and Pospíšil, 2013). Furthermore, SL analogs might be applied to regulate shoot branching or to modulate root structure (Fukui et al., 2013). Given the wide range of potential applications for SLs, low-cost SL analogs with simple structures are needed.

Previously, we showed that carlactone exerts activities of canonical SLs by suppressing the high-tillering phenotype of the SL-deficient rice mutant \textit{d10} and inducing seed germination in \textit{Striga} seeds (Alder et al., 2012). Herein, we describe the design of a new compound, Nitro-Phenlactone (N-PL) (Figure 1A), which is based on the relatively simple structure of carlactone, and that was synthesized in four steps yielding a racemic mixture consisting of a 5’R- and a 5’S-configured stereoisomers (Supplemental Figure 1, 2). N-PL has a shorter chain length than carlactone and contains, instead of a β-ionone ring, a phenyl ring furnished with a nitro-group. The latter differences are thought to improve the stability and solubility.

To assess stability of rac-N-PL and to compare it with that of rac-GR24, we dissolved defined amounts in aqueous solutions adjusted to different pH values (6-8) and used HPLC to determine remaining concentrations in aliquots taken at indicated time points. The stability of both compounds decreased with increased pH (Figure 1B). Compared to rac-GR24, rac-N-PL was slightly less stable at pH 6 and 7, but showed relatively higher stability at pH 8 (Figure 1B).

To evaluate the activity of rac-N-PL in inducing seed germination of root parasitic plants, we applied it at a broad range of concentrations to pre-conditioned seeds of \textit{S. hermonthica} (Figure 1C) and \textit{P. ramosa} (Figure 1D), using rac-GR24 for comparison. rac-N-PL stimulated \textit{S. hermonthica} germination at 10 µM (Figure 1C), while rac-GR24 efficiently stimulated \textit{S. hermonthica} germination at 0.001 µM (Figure 1C). This shows that N-PL has a weak activity in stimulating \textit{S. hermonthica} germination. However, rac-N-PL significantly stimulated seed germination of \textit{P. ramosa} at 0.0001 µM, which was comparable to that of rac-GR24 (Figure 1D). These results suggest that the efficiency of rac-N-PL in inducing seed germination varies depending on the parasitic weeds species.
Such species-dependent differences may reflect the structural diversity of receptors involved in binding and perception of germination stimuli. Recent investigation of SL perception in *S. hermonthica* revealed the presence of KAI2 homologs that act as SL receptors with different binding activities (Tsuchiya et al., 2015). The remarkably high activity of N-PL in inducing seed germination in *P. ramosa* demonstrates the potential of this compound for application in combating roots parasitic weeds, particularly *Phelipanche* species. Moreover, this activity clearly distinguishes N-PL from debranone, an easy to synthesize SL-mimic that, however, has very weak activity in inducing seed germination in root-parasitic plants and, hence, cannot be used as a stimulant of suicidal germination (Fukui et al., 2013).

Next, we tested the phytohormone activities of rac-N-PL by evaluating its effect on tillering and plant height in rice using wild type (Shiokari), SL-deficient (*d10*) and SL-insensitive (*d3*) mutants. Plants were grown in a hydroponic culture system supplied with rac-N-PL (2.5 µM) or rac-GR24 (1 µM) for three weeks. The two compounds led to significant (*P<0.001*) reduction in the number of tillers in *d10* but not in *d3* or WT (Figure 1E). In addition, rac-N-PL- or rac-GR24-treated plants rescued the dwarf phenotype of *d10*, leading to a significant (*P<0.01*) increase in the height of this mutant (Figure 1F). We did not detect any effect in *d3* mutant, suggesting that rac-N-PL has a D3-dependent SL activity in determining tiller number and plant height in rice.

In Arabidopsis, carlactone is converted by the biosynthetic enzyme MAX1 before exerting SL activities (Abe et al., 2014). To test the effect of rac-N-PL on shoot branching and to determine whether the activity is MAX1-dependent, we treated the biosynthetic mutants *max1* and *max4*, and the SL perception mutant *d14* with this compound in hydroponic assays, using rac-GR24 as a positive control. Application of rac-GR24 (1 µM) led to the expected rescue of the branching phenotype in *max1* and *max4* mutant but not in *d14* (Figure 1G). rac-N-PL (2.5 µM) showed a similar effect on rosette branching in *max4* and no effect in *d14*. rac-N-PL also reduced the number of branches in *max1*, however, to a significantly (*P<0.01*) lower extent than in the *max4* mutant. These findings suggest that rac-N-PL can regulate shoot branching in
Arabidopsis in a D14-dependent manner and that the presence of MAX1 increases the activity of N-PL. Hence, rac-N-PL is probably converted by MAX1 into a more active compound. Based on this assumption, N-PL derivates might be developed to have much higher activities in regulating shoot branching. In contrast to shoot branching, rac-N-PL did not affect lateral root density (Supplemental Figure 3). The basis for tissue-dependency of these rac-N-PL effects is unclear; however, this difference reveals the possibility of targeted design of compounds for particular modulations of plant architecture.

We also tested the activity of rac-N-PL in inducing hyphal branching in the AM fungus Gigaspora rosea (Figure 1H). rac-N-PL showed a moderate but significant activity ($P<0.05$) at 0.001 $\mu$M concentration, but no effect at higher concentrations, while rac-GR24 showed expected induction at 0.1 $\mu$M. It may be that the N-PL hyphal branching inducing activity observed at this low concentration is beneficial due to the known positive effects of AM symbiosis. However, a conclusion on the impact of N-PL application on mycorrhization requires further investigations.

A study on the impact of stereochemical configuration on biological activities demonstrated that natural SLs - with 2'C atom in $R$ configuration - inhibit hypocotyl elongation in Arabidopsis, preferentially through the D14-MAX2 signalling pathway, while the effects of karrikins and ent-GR24 (2'C atom in $S$ configuration) on hypocotyl elongation and their induction of germination in dormant Arabidopsis seeds are mediated by the KAI2-MAX2 pathway (Scaffidi et al., 2014). To explore whether rac-N-PL exerts karrikin-like function in regulating seedling development, we examined the effect of two different concentrations (1 $\mu$M and 5 $\mu$M) of this compound on hypocotyl length. Application of rac-N-PL led to hypocotyl length reduction, ranging from 50% at 1 $\mu$M to 72% at 5 $\mu$M (Figure 1I). rac-GR24 showed higher inhibition, leading to about 64% length reduction at 1 $\mu$M concentration (Figure 1I). At the transcriptional level, rac-N-PL changed expression of SL- and karrikin-responsive genes such as DLK2, STH7 and IAA6 (Supplemental Figure 4). rac-N-PL acts via both D14-MAX2 and the KAI2-MAX2 signalling pathways, as suggested by analysis of the response in perception mutants.
We also tested whether rac-N-PL can induce the germination of primary dormant Arabidopsis seeds. However, we did not observe a germination effect (Supplemental Figure 6). The absence of the germination activity might be due to an insufficient uptake of the compound by the seeds or by a lack of an enzymatic activity needed to convert N-PL in a compound with karrikin effects.

Taken together, N-PL is a very promising carlactone-based SL-analog that can be used to combat roots parasitic weeds. Moreover, N-PL will open up possibilities for developing new compounds with specific SLs functions and growth effects.

REFERENCES


Figure 1. Characteristics of N-PL.

(A) Structure of N-PL. A-, B-, C- and D-ring are indicated in 5-Deoxystrigol.

(B) Stability analysis of rac-N-PL at different pH values. Data are means ± SE (n=3).

(C and D) Effect of rac-N-PL on seed germination of S. hermonthica (C) and P. romosa (D). Data are represented as means ± SE from three replicates (P<0.05, Tukey’s HSD test). (E, F and G) Effect of rac-N-PL on tillering (E) and plant height in rice (F), and on shoot branching in Arabidopsis (G). rac-N-PL and rac-GR24 were applied at a concentration of 2.5 µM and 1 µM, respectively. Values ± SE are from 10 plants (P<0.05, Tukey’s HSD test).

(H) Effect of rac-N-PL on the number of hyphal apices branches in G. rosa. Data are represented as the mean number of newly formed apices ± SE (P<0.05, Mann-Whitney’s comparison test).

(I) Effect of rac-N-PL on hypocotyl elongation in Arabidopsis. Data are represented as the mean length ± SE (P<0.01, one way ANOVA).

All GR24 and N-PL used in our experiments are racemic mixtures.

SUPPLEMENTAL INFORMATION

Supplemental information is in attached file.

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AUTHOR CONTRIBUTIONS


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