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New Strigolactone Analogs as Plant Hormones with Low Activities in the Rhizosphere

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ABSTRACT Strigolactones (SLs) are known not only as plant hormones, but also as rhizosphere signals for establishing symbiotic and parasitic interactions. The design of new specific SL analogs is a challenging goal in understanding the basic plant biology and is also useful to control plant architectures without favoring the development of parasitic plants. Two different molecules (23 (3′-methyl-GR24), 31 (thia-3′-methyl-debranone-like molecule)) already described, and a new one (AR36), for which the synthesis is presented, are biologically compared with the well-known GR24 and the recently identified CISA-1. These different structures emphasize the wide range of parts attached to the D-ring for the bioactivity as a plant hormone. These new compounds possess a common dimethylbutenolide motif but their structure varies in the ABC part of the molecules: 23 has the same ABC part as GR24, while 31 and AR36 carry, respectively, an aromatic ring and an acyclic carbon chain. Detailed information is given for the bioactivity of such derivatives in strigolactone synthesis or in perception mutant plants (pea *rms1* and *rms4*, *Arabidopsis max2* and, *max4*) for different hormonal functions along with their action in the rhizosphere on arbuscular mycorrhizal hyphal growth and parasitic weed germination.

Key words: strigolactones; synthetic analogs; *Pisum sativum*; *Arabidopsis*; root parasitic plants; *Gigaspora rosea*; plant growth regulator.

INTRODUCTION

Strigolactones (SLs) are the most recent class of hormones identified in plants initially for their role in the control of shoot branching, especially studied in pea, *Arabidopsis*, petunia, and rice (Gomez-Roldan et al., 2008; Umehara et al., 2008; Brewer et al., 2013). They are mainly biosynthesized in the lower parts of the stem and roots, after which they are presumably transported in a root-to-shoot direction to other shoot parts (Kohlen et al., 2011). Following their initial discovery as shoot branching regulators, SLs were shown to be involved in nodule formation (Soto et al., 2010; Foo and Davies, 2011), root architecture (Kapulnik et al., 2011; Ruyter-Spira et al., 2011), adventitious root formation (Kohlen et al., 2012; Rasmussen et al., 2012), stimulation of cambium activity (Agusti et al., 2011), and also in stem elongation (de Saint Germain et al., 2013b). In addition, SLs

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promote thermoinhibited seed germination of non-parasitic plants (Toh et al., 2012). Consequently, it seems that, just like other plant hormones, SLs are involved in many aspects of plant development. In addition, these carotenoid-derived compounds were already known for their roles as allelochemicals in symbiotic and parasitic interactions in the rhizosphere: SLs belong to a class of compounds first identified in 1966 as stimulants of the seed germination of parasitic weeds *Orobanche*, *Phelipanche*, and *Striga* (Cook et al., 1966; Xie et al., 2010). These parasitic plants are major agricultural pests found around the Mediterranean Sea and in sub-Saharan Africa, respectively, where they constitute the major cause of crop damage. Because of their germination activity for parasitic seeds, SLs and analogs have been developed as soil treatments through suicidal germination (Kgosi et al., 2012; Zwanenburg and Pospisil, 2013). In conditioned seeds of *Phelipanche ramosa*, SLs induced catabolism of abscisic acid for germination (Lechat et al., 2012). They are produced mainly in roots in very small amounts (pg-scale/plant/day) and are excreted in the rhizosphere (Yoneyama et al., 2012). They also stimulate spore germination and hyphal proliferation of arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005; Besserer et al., 2006), and were also found to have an effect on phytopathogenic fungi (activation or inhibition) (Dor et al., 2011). During AM symbioses, plants receive water and mineral nutrients from their fungal partners, hence promoting optimal plant growth conditions (Reinhardt, 2007). SLs are part of a molecular dialog between plants and AM fungi, which is now beginning to be understood (Maillet et al., 2011; Genre et al., 2013; Nadal and Paszkowski, 2013). SLs are also produced by the moss *Physcomitrella patens*, where they act as signaling factors for controlling plant extension and potential interaction with neighbor individuals as a quorum sensing-like signal. This emphasizes the roles of SLs in both plant development and communications between organisms (Proust et al., 2011).

The structural core of SLs is a tricyclic lactone (ABC-rings), with various substitution patterns on the AB-rings, connected via an enol ether linkage to an invariable α, β-unsaturated furanone moiety (D-ring) (Figure 1A and 1B). To date, at least 19 natural SLs have been identified and completely characterized in root exudates. They can be divided into two families according to a recent structural revision: one in which the stereochemistry in the BCD part is the same as (+)-strigol and the other in which the stereochemistry in the BCD part is the same as (–)-orobanchol (Xie et al., 2013).

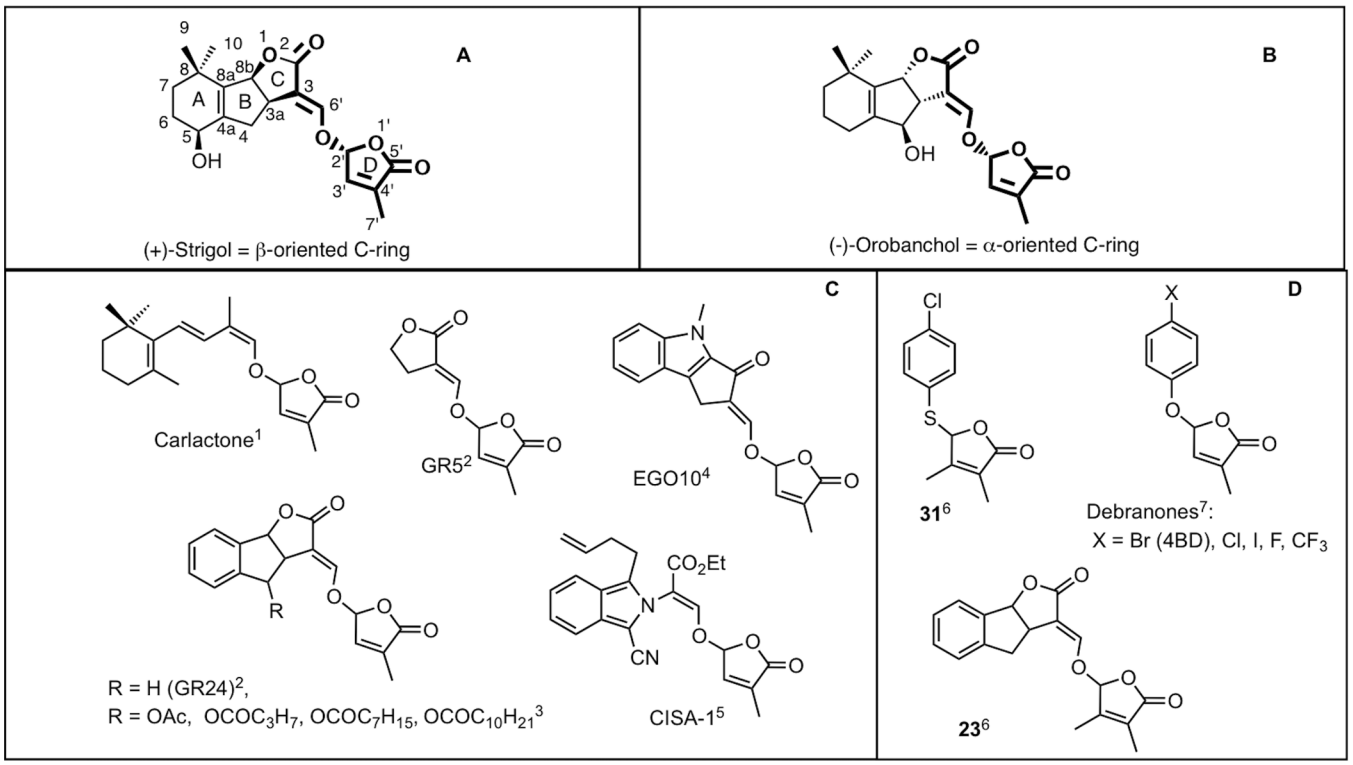

Figure 1. Natural SLs and SL Analogs Active as Plant Hormones.

(A) SL family with BCD stereochemistry as in (+)-strigol.

(B) SL family with BCD stereochemistry as in (–)-orobanchol.

(C) Strigolactone analogs and precursor described as plant hormones.

(D) Strigolactone analogs described as selective plant hormones and with low activity as root-parasitic weed stimulants. The bold chemical bonds correspond to the invariable part in the natural SLs. Ac, acetyl; Me, methyl; [1] Alder et al., 2012; [2] Gomez-Roldan et al., 2008; [3] Chen et al., 2013; [4] Cohen et al., 2013; [5] Rasmussen et al., 2013; [6] Boyer et al., 2012; [7] Fukui et al., 2011, 2013.

The biosynthesis of SLs, largely studied thanks to mutants in pea (*rms* (*ramosus*)), *Arabidopsis* (*max* (*more axillary branches*)), rice (*d* (*dwarf*)), and petunia (*dad* (*decreased apical dominance*)) (Supplemental Figure 1), involves isomerization of β-carotene by a β-carotene isomerase (D27), and cleavage at the C9′, C10′ position by Carotenoid Cleavage Dioxygenase 7 (CCD7) to form 9-*cis*-β-apo-10′-carotenal. Oxidation and intramolecular rearrangement by CCD8 lead to carlactone (Figure 1C and Supplemental Figure 1). Bioactive carlactone, very recently isolated (Alder et al., 2012), is central for understanding the biosynthesis of SLs and also for the development of SL analogs as shown in our present work. Carlactone is highly unstable, to date still not detected *in planta* (Alder et al., 2012; K. Yoneyama, personal communication) and less bioactive than SLs (Alder et al., 2012; Scaffidi et al., 2013). The biosynthetic pathway implies that the A and D-rings are first made followed by the formation of the BC-ring to give 5-deoxystrigol (and stereoisomers) via putative(s) cytochrome P450 (MAX1?) enzyme(s). Analysis of branching mutants that no longer respond to the SL application (*max2/rms4/d3*, *Atd14/d14/dad2*) let to the identification in petunia of DAD2 as a α/β-hydrolase catabolizing SL (Hamiaux et al., 2012). X-ray crystallography studies confirmed that DAD2, AtD14, and D14 belong to the α/β hydrolase fold family (Kagiyama et al., 2013; Zhao et al., 2013). DAD2 was shown to bind to one of the two petunia MAX2 homologs, PhMAX2A, an F-box type protein required for SL signaling in a SL dependent fashion, suggesting a novel mechanism-of-action that involves SL hydrolysis (Hamiaux et al., 2012; Scaffidi et al., 2012).

The great importance of SLs in plant chemical biology with opposite effects (repression of cell division in axillary bud and stimulation of cambial activity/stem elongation/seed germination or hyphal proliferation) depending on the target, their extremely low bio-availability, and the difficulties to obtain natural SLs by organic synthesis due to long multistep syntheses (Humphrey et al., 2006; Shoji et al., 2009; Chen et al., 2010; Kitahara et al., 2011) prompted us to develop new SL analogs. The objective of this study was to obtain analogs easily accessible in sizeable quantities, more stable, and with similar bioactivity to natural SLs. One major aim was also to obtain specificity according to the target function in contrast to the reference compound in all bioassays, the synthetic aromatic SL analog GR24 (Figure 1C), which possesses general high bioactivity. Specific SL analogs were targeted because they can teach more about SL bioactivity/stability/perception.

GR24, known for a long time (Johnson et al., 1981), was initially developed for its high germination activity and its increased stability compared to natural SLs. Indeed, natural SLs are particularly sensitive in natural media resulting from a Michael addition of water on the enol ether linkage (Akiyama et al., 2010). Moreover, GR24 is accessible by organic synthesis on a multi-gram scale (Mangnus et al., 1992). The bioactiphore for the germination of parasitic weeds has been extensively studied since its discovery in the 1970s and was found to reside in the CD part (Zwanenburg and Pospisil, 2013). Essential structural features for proliferation of AM fungi hyphae appear to be similar to the requirements for parasitic seed germination, but important SL structure variations seem to affect bioactivity (Akiyama et al., 2010; Prandi et al., 2011). Contrary to the seed germination of parasitic weeds, the oxygenation pattern of the SL and the replacement of the enol ether link between rings C and D by an alkoxy or an imino ether have little effect on its activity on AM fungi. The presence of the ABC-rings also seems essential for proliferation of AM fungi hyphae (de Saint Germain et al., 2013a).

Few studies have been described for the hormonal function of SLs (Gomez-Roldan et al., 2008; Umehara et al., 2008; Fukui et al., 2011; Cohen et al., 2013; Fukui et al., 2013). We recently reported the first structure–activity relationship (SAR) study in pea of SLs as a plant hormone controlling shoot branching (Boyer et al., 2012). In this study, we established that the presence of a Michael acceptor as well as a methylbutenolide or a dimethylbutenolide motif in the same molecule is essential for activity. We demonstrated that SLs show potent activity for the control of shoot branching in a structure-dependent manner but with low specificity as drastic ABC-ring substitutions or modifications are tolerated (see SL analogs described as active as plant hormones; Figure 1C and 1D) (Boyer et al., 2012; Chen et al., 2013; Rasmussen et al., 2013).

Here, we report a complete study concerning the biological activity of the most active SL analogs recently described for the branching inhibition in pea (Boyer et al., 2012). In addition, a new class of SL analogs (AR series) is also evaluated in various species: vascular plants (functions in shoot and root), parasitic weeds, AM fungi.

RESULTS

Development of New Specific SL Analogs as Plant Hormones: Chemical Stability

In a previous SL SAR study for the inhibition of bud outgrowth in pea, we reported high activity for both **23** (3′-methyl-GR24) (Boyer et al., 2012) (SL analogs first generation) and **31** (thia-3′-methyl-debranone-like molecule) (Boyer et al., 2012) (SL analogs second generation) compounds by systematically simplifying the SL skeleton or adding substituents to the D-ring (Figure 1D). The activity of carlactone on tillering in rice (Alder et al., 2012)—its lower activity than GR24 as a germination stimulant—prompted us to develop a third generation of SL analogs bearing a D-ring connected by an enol ether bond to an acyclic unsaturated system. Starting from known derivatives 5-chloro-3,4-dimethylfuran-2(5*H*)-one (Canevet and Graff, 1978) and methyl malondialdehyde sodium salt easily synthesized in large quantities (Nair et al., 1981), we performed the coupling in DMSO to improve the solubility of the sodium salts furnishing AR8 in high yield (91%). AR36 was obtained by Wittig reaction with methyl (triphenylphosphoranylidene)acetate in boiling toluene in 61% yield (80% after recovery of the starting aldehyde). It has to

be mentioned that this reaction does not occur at lower temperatures (<70°C) and that AR36 is subject to degradation if the reaction time is extended. The highest yield was obtained in refluxing toluene for 12 h. The *E*-geometry of the AR36 enol ether was determined by NOESY correlation observed between H^5 and H^3 (Figure 2), while the *E*-geometry of the enone was evident from the value of the coupling constant between H^2 and H^3. The analog AR32 was synthesized from AR8 by Wittig methylenation using methylenetriphenylphosphorane at low temperature (–78°C to –20°C) in moderate yield (38%).

Due to the fact that the activity differences between SL analogs may be attributed to their instability in protic medium, we performed a stability test as previously described in an aqueous solution ($EtOH/H_2O$) (Boyer et al., 2012). The stability of the SL analogs was in order AR36 > **31** > **23** >> GR24 in aqueous solution (Figure 3). Hence, we developed new SL analogs with easy access by organic synthesis and high stability in an aqueous solution compared to the well-known GR24 and natural SLs (Boyer et al., 2012).

SL Analogs as Bud Outgrowth Inhibitors in Pea

A simple bioassay for monitoring SL activity for inhibiting shoot branching was developed (Gomez-Roldan et al., 2008; Boyer et al., 2012) using the high branching SL-deficient *rms1* mutant in garden pea. Direct application of 10 µl of the solution to be tested on an axillary bud at a given node (generally node 3 or 4) was performed before its outgrowth and the bud/branch length was measured 8–10 d later. The different compounds were first tested at a concentration of 1 µM and lower concentrations were tested to analyze quantitative differences in bioactivity between molecules. All

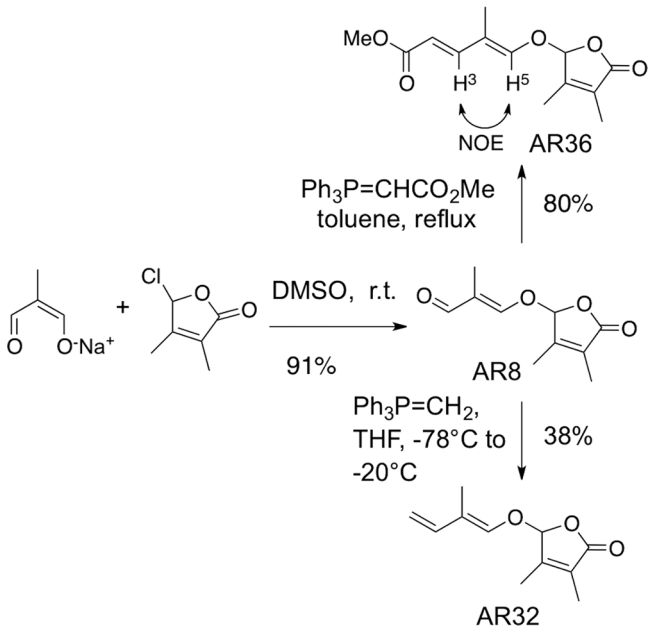

Figure 2. Chemical Synthesis of AR Series.

tested SL analogs showed significant activities at a concentration of 1 µM (Figure 4A) but AR8 and AR32 were found to be significantly less active than AR36 and GR24 at a concentration of 1 µM (AR32 versus GR24 1 µM, P = 5.076e-06; AR8 versus GR24 1 µM, P = 2.428e-06; Kruskal-Wallis rank sum test) and 100 nM (AR32 versus GR24 100 nM, P = 3.214e-07; AR8 versus GR24 100 nM, P = 1.928e-05; Kruskal–Wallis rank sum test). The same dose-dependent activity as for GR24 was found for our bioactive SL analogs. A limit of activity was shown at a concentration of 1 nM at node 3. At node 4, while GR24, **23**, and **31** were still active, at a concentration of 0.1 nM, these SL analogs were not significantly active or barely significant (Supplemental Figure 2) (GR24 versus CTL 0, P = 0.42; **23** versus CTL 0, P = 0.1417; **31** versus CTL 0, P = 0.04531; Kruskal–Wallis rank sum test). CISA-1, recently described as the first fluorescent SL analog active as a plant hormone (Rasmussen et al., 2013), was found to be slightly less active than **23** and **31** at 100 nM (**23** versus CISA-1; P = 0.0454; **31** versus CISA-1; P = 0.003573, Kruskal-Wallis rank sum test) and less active than **23**, **31**, and AR36 at 10 nM (**23** versus CISA-1; P = 0.00019; **31** versus CISA-1; P = 0.0017; AR36 versus CISA-1; P = 0.044, Kruskal–Wallis rank sum test) (Figure 4B). At 1 nM, CISA-1 was found to be significantly less active than AR36 (AR36 versus CISA-1; P = 0.04318, Kruskal–Wallis rank sum test) while no significant differences were observed with the other analogs (e.g. **31** versus CISA-1; P = 0.055, Kruskal–Wallis rank sum test). On the contrary, the analogs (**23**, **31**, AR36) were clearly more active than 4-Br debranone (4BD) (Fukui et al., 2013) (Figure 1D) at 100 nM and 10 nM (Figure 4B) (e.g. 10 nM at node 3; AR36 versus 4BD; P = 0.006277; Kruskal–Wallis rank sum test). CISA-1 was observed to be more active than 4BD only at 100 nM (CISA-1 versus 4BD, 100 nM at node 3; P = 0.0003839; 10 nM at node 3; P = 0.1003; Kruskal–Wallis rank sum test).

Effects of SL Analogs under Hydroponic Culture Conditions in Pea: Action on Shoot Branching and on Plant Height

To investigate the effects of our most active SL analogs (AR36, **23, 31**) and the reference molecule GR24 on shoot branching when provided via the roots, we developed a hydroponic culture system in which pea seedlings were placed 6 d after sowing (before leaf expansion at node 3). SL analogs were added at this stage (1 µM) into the culture solution and branching at each node together with plant height were measured 2 weeks later. Bud outgrowth was significantly repressed at the upper nodes (nodes 3 to 4) by treatment with any of the 4 SL analogs (Figure 5A and Supplemental Figure 3). AR36 and **23** (3′-Methyl-GR24) were significantly more active than GR24 and **31** in inhibiting branching at these nodes (AR36 versus **31**, P = 0.00011; AR36 versus GR24, P = 0.016; **23** versus **31**, P = 0.000256; **23** versus GR24, at the limit of significance, P = 0.05813; Kruskal–Wallis rank sum test). Basal branching

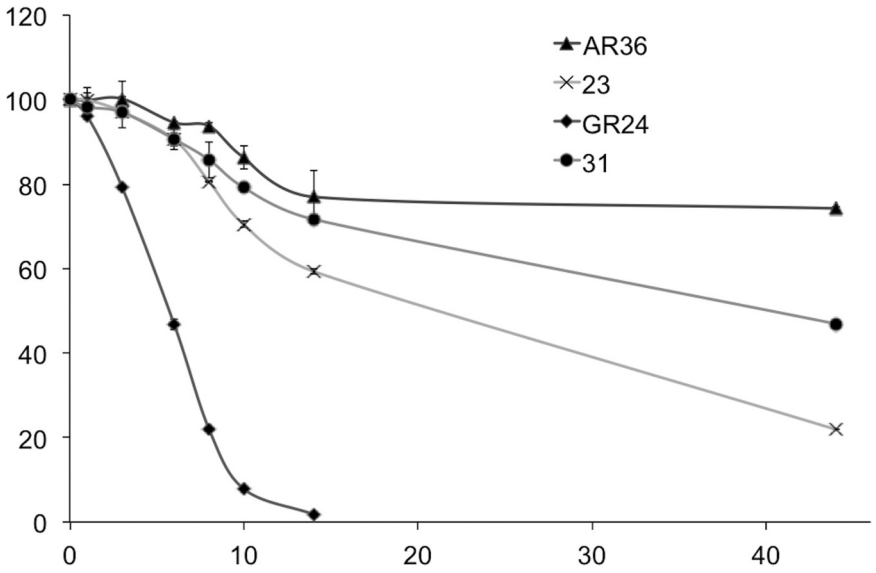

Figure 3. Chemical Stability of GR24, AR36, 23, and 31 in an ethanol:water (1:4, v/v) solution at 21°C (pH 6.7). Data are means ± SE (n = 3). X axis (time (d)); y axis (% substrate).

of dwarf gibberellin deficient pea lines is generally very vigorous (Murfet and Reid, 1993). At basal nodes (nodes 1 and 2), two branches per node were generally observed for our SL-deficient *rms1* mutant line (Supplemental Figure 3). The length of the different basal branches was measured and analyzed separately in the *rms1* mutant grown in hydroponics. A significant reduction of the bud/branch length was observed only for the second branch at both nodes for **23** and AR36 and only for the second branch at node 2 for GR24 and **31**. In this experiment, it is interesting to note than only compound AR36 significantly reduced the growth of the main branch at node 1. Consequently, experiments in which SL analogs were applied via the roots support strigolactone-like branching inhibition activity as well as acropetal transport for compounds AR36, **31, 23**. Moreover, AR36 and **23** showed higher bioactivity than did GR24 and **31** when applied through roots.

It was demonstrated that the dwarfism of the pea SL-mutants was not due to high branching and SLs were shown to stimulate internode length when applied by the roots (de Saint Germain et al., 2013b). Plant height was measured after application of the four analogs and all of them significantly stimulated plant height with AR36 and particularly **23** showed the strongest activity in comparison to GR24 and **31** (Figure 5B). Consequently, both analogs showing the highest bioactivity for inhibiting branching also exhibited the strongest bioactivity for stimulating internode elongation.

Adventitious Root and Lateral Root Formation in *Arabidopsis* Are Inhibited by the SL Analogs

In *Arabidopsis*, the SL-deficient *max4* as well as the SL-response *max2* mutants produce significantly more lateral roots compared to WT (Rasmussen et al., 2012) (Supplemental Figure 4). Similarly to GR24, application of AR36, **31**, **23** also inhibited adventitious root formation and this was observed in the same concentration range as for bud outgrowth inhibition in pea (1–1000 nM) (Supplemental Figure 4 and Figure 6). For both Columbia wild-type and *max4*, GR24 and **23** caused a significant decrease in the number of adventitious roots from 1 nM. In wild-type, treatment with **23** was similar to GR24 at 1 and 100 nM concentrations while, at 10 and 1000 nM, it was less effective in inhibiting adventitious roots as compared to GR24. In *max4*, **23** was also less effective at inhibiting adventitious roots at 10 nM, while, at higher concentrations, it was as effective as GR24. For Columbia wild-type, all compounds caused a significant decrease in the number of adventitious roots from 10 nM, while, for *max4*, all adventitious rooting was significantly decreased after just 1 nM treatment with any compounds.

In wild-type, treatments with **31** and AR36 were similar to GR24 at 1 and 10 nM concentrations, while, at higher concentrations, they were less effective in inhibiting adventitious roots as compared to GR24. In *max4*, **31** and AR36 were also less effective at inhibiting adventitious roots. At 1 nM, AR36 was already significantly less inhibitory, while **31** activity was not different from that of GR24. All compounds showed similar levels of activity at 10 nM, while, at 100 nM, both **31** and AR36 were significantly less effective than GR24. Both compounds were also less effective than GR24 at 1000 nM.

The effects of SL on root architecture have been recently demonstrated (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Therefore, we analyzed lateral root development in *A. thaliana*, 9 d after treatment. As expected and in accordance with the adventitious root bioassay, no inhibition of lateral root

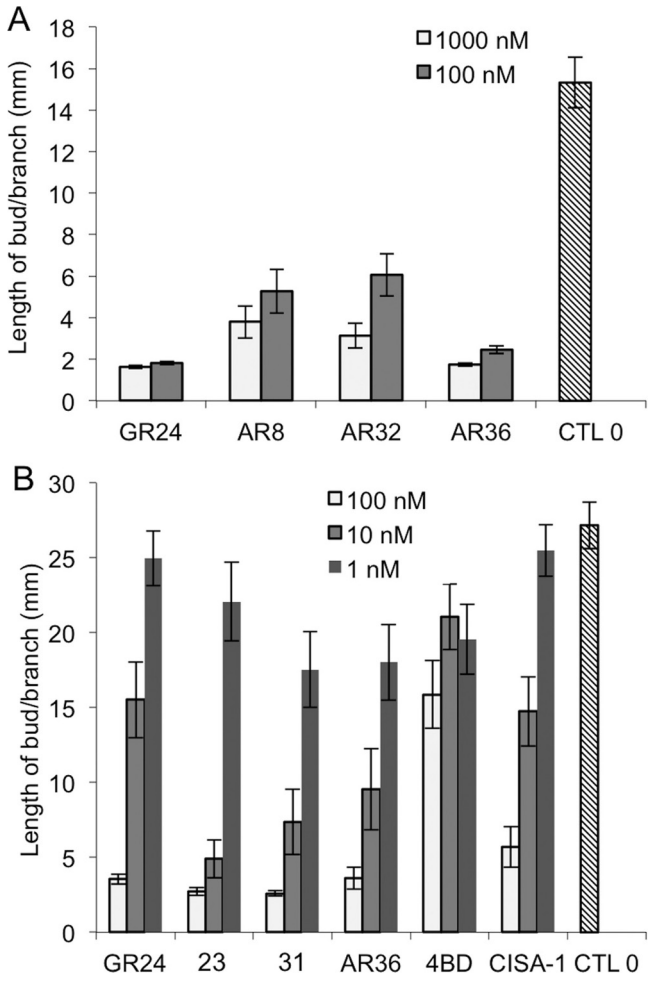

Figure 4. Bioactivity of SL Analogs for the Control of Branching in Pea.
(A) Activity of AR series, 31 and 23. Length of axillary bud of *rms1* pea plants, 8 d after direct application of a solution at 1 µM and 100 nM at node 3. Data are means ± SE (n = 24).
(B) Dose-dependent activity (100–1 nM) of the SL analogs GR24, 23, 31, 4BD, CISA-1 for branching inhibition. Length of axillary bud of *rms1* plants, 8 d after direct application at node 3. Data are means ± SE (n = 10–24). CTL 0, control 0.

formation was observed on the *max2* mutant (SL-response mutant) with any of the SL analogs (Figure 7). Moreover, as reported, *max2-1* mutants displayed a higher lateral root density at 9 d than did Columbia WT plants (Figure 7). GR24 as well as AR36, **31**, and **23** inhibited lateral root formation of WT in the same range but **31** proved to be inactive in these conditions (Figure 7).

SL Analogs Are Not Phytotoxic and Use the Same SL Signaling Pathway as Natural SLs for Plant Hormone Activities

In pea, SL signaling for inhibition of shoot branching involves the F-box protein RMS4 and the TCP transcription factor PsBRC1 (Brewer et al., 2013). *PsBRC1* is strongly expressed in axillary buds and transcript levels of *PsBRC1* are up-regulated in axillary buds by an exogenous SL application (Boyer et al., 2012; Braun et al., 2012). The activity of SL analogs which showed similar bioactivity for bud outgrowth inhibition can be tested using this molecular marker to confirm that SL analogs use the same signaling pathway as do natural SLs to repress branching. *PsBRC1* transcript levels were quantified using real-time PCR in *rms1* axillary buds 6 h after direct application of GR24, CISA-1, **23**, **31**, and AR36 at a concentration of 1 µM (Figure 8). AR36 and CISA significantly increased *PsBRC1* transcript levels. **23** and GR24 increased transcript levels but this increase was barely significant for GR24 and not at all significant for **23** (only two biological repeats for this analog).

Similarly, to confirm that SL analogs inhibit branching via the RMS4 F-box protein, the molecules GR24, **23**, **31**, and AR36 were tested on the SL-response mutant *rms4* at two different concentrations (3000 nM and 100 nM) (Supplemental Figure 5A). No significant branching inhibition was observed for GR24 or for the three other SL analogs at both concentrations. The same test was performed with CISA-1 at 1000 nM giving the same results (Supplemental Figure 5B). As expected with the *max2* mutant (*Arabidopsis* SL-response mutant), no inhibition of adventitious root formation was also observed (Supplemental Figure 4). We conclude that our SL analogs together with GR24 and CISA-1 repress axillary bud outgrowth via RMS4 and PsBRC1. The fact that these SL analogs cannot inhibit branching of the *rms4* response mutant also indicates that they are not phytotoxic at the tested concentrations.

The Effects of SL Analogs in the Rhizosphere

SL Analogs Are Moderate to Low Inducers of Orobanche (Phelipanche) and Striga Germination

The germination stimulatory activities of SLs in *Orobanche* and *Striga* were extensively studied in the past with the range of half maximal effective concentrations (EC_{50}) (10^{-10}–10^{-15} M) varying according to the root parasitic plants (Kim et al., 2010; Malik et al., 2011). EC_{50} reflects the affinity of the compound to the putative receptor over the effectiveness of the response induced. In this study, the EC_{50} of each compound (GR24, AR36, **23**, **31**, CISA-1) was determined with a range of concentrations varying between 10^{-13} M and 10^{-5} M and the maximum percentage of germination. Seeds of *P. ramosa* (Pomel), *Orobanche cumana*, *Orobanche minor*, and *Striga hermonthica* were used. The maximum germination percentage varied from one parasitic plant to another, such as for GR24 from 89% ± 6% to 57% ± 5% (e.g. *P. ramosa* pv C to *S. hermonthica*) (Supplemental Table 1) and all four analogs exhibited similar (**23**) or lower activities compared to the GR24. Activities of SL analogs were then compared using their EC_{50}. Interestingly, the highest activity was observed with GR24 for all tested species of parasitic plants (EC_{50} ± SE = 4.2×10^{-12} ± 1.2×10^{-12} mol L^{-1} (*P. ramosa* pv C) to 1.4×10^{-8} ± 5.7×10^{-9} mol L^{-1} (*Orobanche*

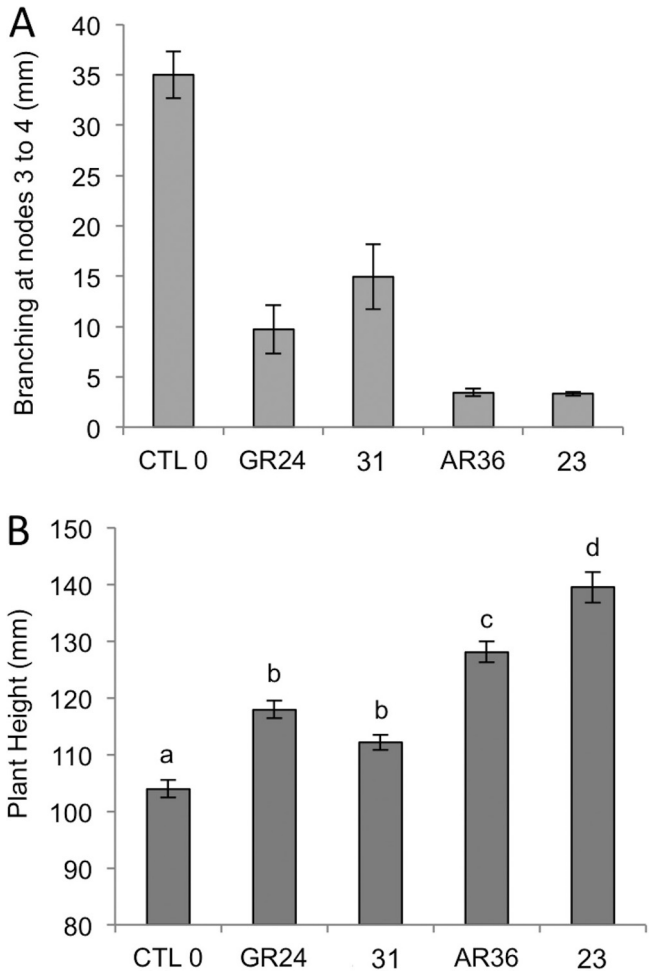

Figure 5. The Most Active SL Analogs for Inhibiting Pea Branching Are Also the Most Active for Stimulating Plant Height.

(A) Branching at nodes 3 to 4 after treatment with SL analogs (1 µM).
(B) Plant height after treatment with SL analogs (1 µM). Data are means ± SE (n = 20). Values that have the same letter (a–d) are not significantly different at the P = 0.05 level (Anova test). CTL 0, control 0.

minor)) (Figure 9 and Supplemental Table 1). For instance, for *P. ramosa* pv C, the analogs **23**, CISA-1, and **31** presented moderate to low activity in comparison with GR24 (about 100, 100 000 times less active, respectively) and AR36 was not active.

SL Analogs Do Not Enhance Branching of the Arbuscular Mycorrhizal Fungal Hyphae

We also tested the activity of our developed SL analogs for hyphal branching using *Gigaspora rosea*: with our SL analogs (**23**, **31**, AR36) at 100 nM, no significant difference with CTL 0 was observed while GR24 was active as previously described (Besserer et al., 2006) (Figure 10). CISA-1 was mildly active. Consequently, in these conditions, our SL analogs (**23**, **31**, AR36) proved to be inactive on AM fungi.

DISCUSSION

Quantitative Differences in Hormonal Activity for SL Analogs According to Developmental Process and/or Species

Numerous SL analogs have been developed since the discovery of this class of compounds (Zwanenburg and Pospisil, 2013). Nevertheless, these compounds have been almost exclusively tested for their effect on parasitic weed seed germination. Only few studies have tested all the hormonal functions of SLs with the exception of GR24, known as the reference compound in SL bioassays. After recently testing various natural SLs and SL analogs for shoot branching inhibition in pea (Boyer et al., 2012), we demonstrated that complex SLs are not necessarily better for higher bioactivity.

Two recent studies reported two SL analogs, CISA-1 and 4BD, as showing plant hormone activity. Rasmussen and co-workers (2013) established that CISA-1, a fluorescent SL analog, structurally close to the Nijmegen-1 (Nefkens et al., 1997) shows higher biological activity than GR24 in the adventitious rooting as well as in the *Orobanche aegyptiaca* seed germination assay, and similar activity in the branching assay in *Arabidopsis*. These effects correlated with a higher stability in an aqueous medium. In addition, 4BD, a new phenoxyfuranone compound has been shown to act as a plant-specific SL mimic (in rice and *Arabidopsis*) with very low activity on seed germination of *S. hermonthica* (Fukui et al., 2013). It is important to note that, in *Arabidopsis*, 4BD reduced lateral root growth but was not as potent as GR24 (Fukui et al., 2013).

Here, the hormonal action of the SL analogs was evaluated using pea (*Pisum sativum*) for shoot branching and plant height and *Arabidopsis* for their effects in roots as recently described in the literature (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). In addition, the SL analogs were also evaluated using an adventitious rooting assay in *Arabidopsis* which proved to be a rapid method for testing biological activity of SL analogs for this function (Rasmussen et al., 2012).

Our analogs revealed bioactivity in pea with a similar range as with CISA-1 but more active than 4BD, which showed poor activity in our conditions (Figure 4C). Globally, the new analogs **23** and AR36 were the most active for repressing branching compared to GR24, CISA-1, and **31** using the direct application method or using hydroponics. In the latter system, **23** and AR36 were also the most active for stimulating plant height. In contrast, **31**, **23**, and AR36 were slightly less active than GR24 for their inhibition of *Arabidopsis* adventitious root formation for which CISA-1 proved to be slightly more active than GR24 (Rasmussen et al., 2013). The debranone-like molecule **31** did not show significant activity for lateral rooting (Figure 7) and this compound was often one of the less active in other bioassays.

We cannot rule out that these slight differences in bioactivity between analogs may have been due to the species used for the bioassay. Consequently, when looking for the most active SL

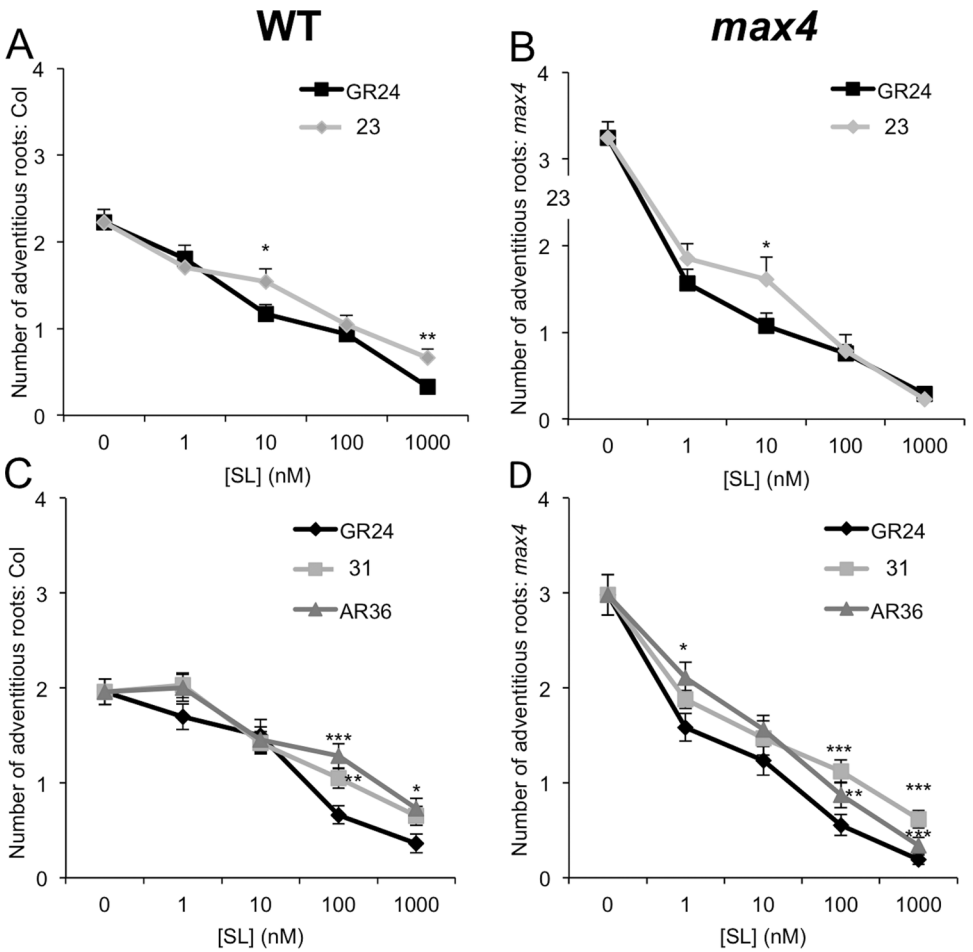

Figure 6. Dose-Dependent Activity (0–1000 nM) of *Arabidopsis* Columbia Wild-Type (wt) (left) and *max4* Mutant (right) to 4 SL Analogs on Adventitious Root Number.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the GR24 treatment of the same concentration (Student's *t*-test). Means are presented ± SE.

analog for a given process in a given plant, it will be essential to test with various bioassays at low concentrations or, at the most, at the 5 µM concentration in accordance with the concentrations of natural SLs found in plant (Yoneyama et al., 2007).

These analogs were tested on the SL-response mutants from pea (*rms4*) and from *Arabidopsis* (*max2*) to confirm that they were inactive on these mutants and hence used the same RMS4/MAX2-dependent signaling pathway as natural SLs (and GR24). Their inactivity on these mutants also indicated that these analogs were not phytotoxic for the axillary bud and for the lateral roots. The increase in *PsBRC1* transcript levels after application of most analogs on the axillary bud further confirmed that the same signaling pathway was used for branching inhibition. This molecular assay can also explain differences of the hormonal activity between SLs and analogs. There is a global correlation between bioactivity for branching inhibition and transcript-level induction: AR36 is more active than **31** as demonstrated in the hydroponic culture. The particularly high induction by AR36 could be due to the particular properties of this compound (e.g. penetration rate in the tissue) independently of the stability in biological media because the *PsBRC1* transcript levels were analyzed as early as 6 h following application.

SL SAR Data: ABC-Rings Are Necessary for the Hyphal Proliferation Activity in AM Fungi, Methyl at C3′ Is Crucial for Specific Endogenous Activities

We have demonstrated that a dimethylbutenolide motif has a boosting effect on the repression of shoot branching at least in pea. This is contrary to the effect on the germination activity (Zwanenburg et al., 2013; Zwanenburg and Pospisil, 2013), where the response depends both on the part of the molecule attached to the D-ring (see Nijmegen and butenolides series) and/or on the parasitic species. We validated that the ABC-part of SLs can be replaced by an aromatic ring (**31**) or an unsaturated acyclic carbon chain (AR36) without changing the bioactivity for the shoot branching inhibition. Nevertheless, the structure–activity relationship demonstrated that the A-ring is important for activity as a branching factor in AM fungi (Besserer et al., 2006; Akiyama et al.,

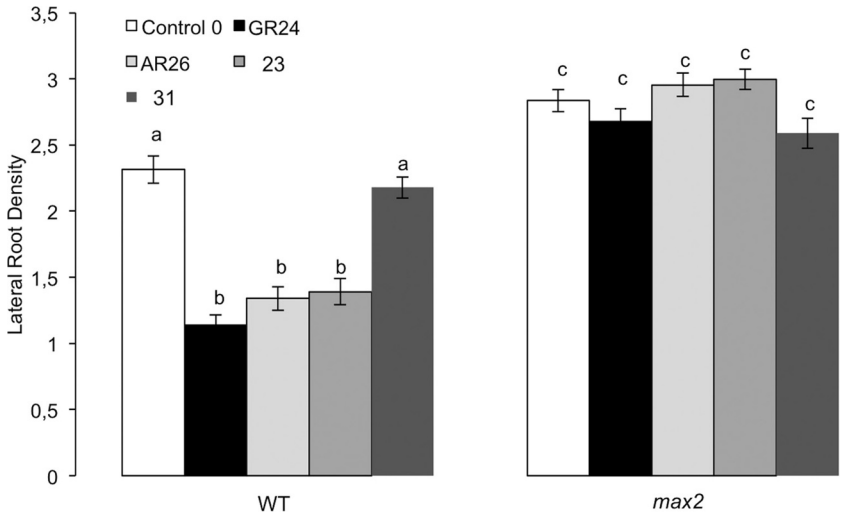

Figure 7. Lateral Root Density (LRD), 9 d, After Mock, GR24, AR36, 23, and 31 Treatment (1 µM) of *Arabidopsis* Columbia Wild-Type (*wt*) (Left) and *max2* Mutant (Right) Roots.

Means are presented ± SE ($n > 28$). Different letters (a–c) represent significantly different means (ANOVA mixed models analyze, $P < 0.05$). CTL 0, control 0.

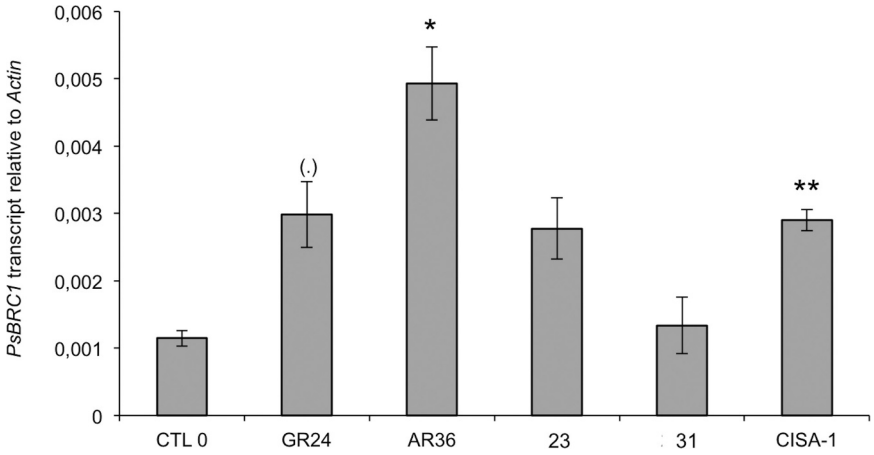

Figure 8. *PsBRC1* Transcript Levels in the Axillary Bud at Node 3 of *rms1* Pea Plants 6 h after Treatment with SL Analogs (1 µM).

Data are means ± SE ($n = 3$ pools of 30 plants). *** $P < 0.001$ relative to the CTL 0 treatment; * $P < 0.05$ relative to the CTL 0 treatment (Student's *t*-tests). CTL 0, control 0.

2010). In our studies, we confirmed this result (no activity for AR36 and **31**, limited effect for CISA-1) and demonstrated that an extra-methyl at the C3′ position has a drastic effect on branching activity in AM fungi (**23** versus GR24). GR24 is the best AM branching factor candidate in comparison with natural SLs or other SL analogs. Our results confirmed that SAR using the AM fungi bioassay is the most specific and that little structural variations have a drastic effect on bioactivity. In contrast, hormonal effect and parasitic seed germination appear to be induced by a wider range of bioactive structures. A major breakthrough in understanding SL perception would be the characterization of SL protein receptors in AM fungi and root parasitic plant seeds. A specific branching factor in AM fungi, analog of the SLs, still needs to be found and would be a valuable target for the future but, to date, difficult to design in accordance with our results and those of Prandi and co-workers (2011). In these studies, the most active compounds for AM fungi hyphal branching are also the most active for root parasitic seed germination.

23, 31, and AR36, Three SL Analogs with Promising Specific Activities as Plant Growth Regulators

In the present study, we have demonstrated that our analogs can be used as plant growth regulators, especially AR36 that shows no phytotoxicity and does not favor the germination of various root parasitic weeds. However, an exhaustive study of the activity of our SL analogs for germination of root parasitic weeds is needed. The perception of SLs was proven very

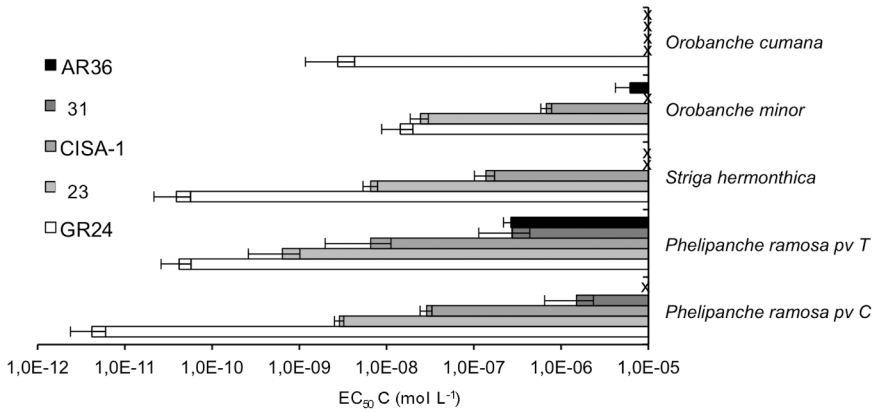

Figure 9. EC_{50} (Half Maximal Effective Concentration) (mol L^{-1}) of of AR36, 31, CISA-1, 23, and GR24 Analogs toward Various Root Parasitic Plant Seeds Germination.

EC_{50} are presented ± SE. The 'x' indicates not measurable.

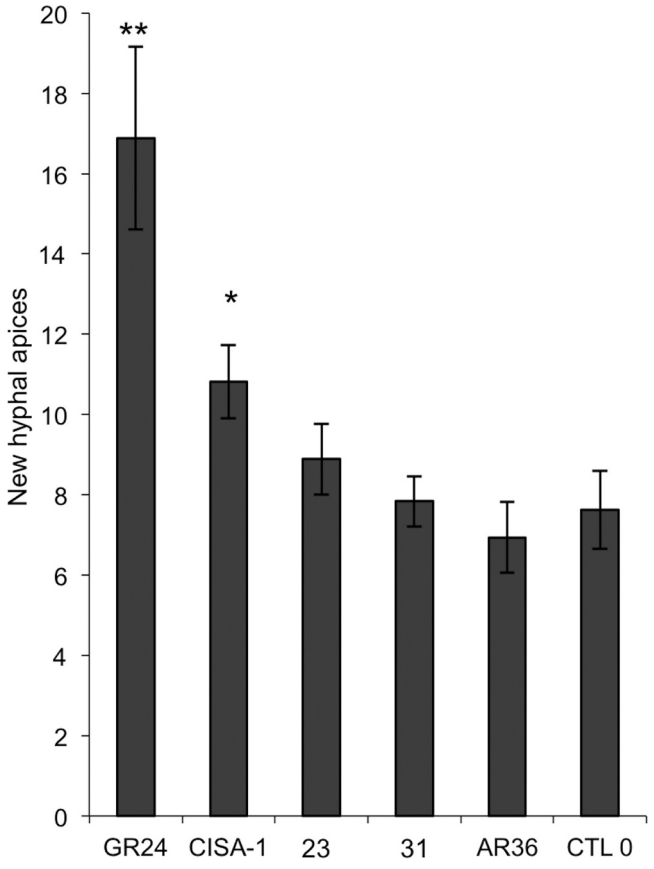

Figure 10. Comparative Activity of GR24, CISA-1, 23, 31, and AR36 for Hyphal Branching of *Gigaspora rosea* after Treatment with SL Analog (100 nM).

Data are means ± SE (n = 15–21). * $P < 0.05$; ** $P < 0.01$ indicates significant difference with CTL 0 (Student's t-test). CTL 0, control 0.

different on each parasite species (Yoneyama et al., 2009), probably in accordance with their specific evolution. For instance, CISA-1 (Rasmussen et al., 2013) was proven to be a potent inducer of *O. aegyptiaca* germination but a lower inducer in the present study.

We confirmed that, in addition to regulating shoot branching (Gomez-Roldan et al., 2008) and various aspects of root development (Kapulnik et al., 2011; Ruyter-Spira et al., 2011), all of our most active SL analogs contributed to the regulation of the main shoot elongation. Until recently, based on the use of dwarf mutants in *Arabidopsis*, pea, rice and maize, auxin, gibberellins, and brassinosteroids were shown to be the key hormones determining internode length (Ross et al., 2011). We recently reported that the relative dwarfism observed in pea SL-deficient and SL-response mutants was not due to their increased branching, which may reduce the amount of resources for main stem growth (de Saint Germain et al., 2013b). We demonstrated that SLs stimulate internode elongation, hence plant height, a major agronomic target in plant breeding for improving seed yield, biomass, and standing ability, independently of gibberellin. All of our SL analogs stimulated internode length in *rms1* plants with **23** and AR36 treatments showing a higher effect in comparison to GR24 and **31**. Undoubtedly, new physiological effects of SLs on shoot architecture will be discovered in the near future. The design of new SL analogs should be possible after the elucidation of the perception of SLs by the presumed receptor proteins as recently found in petunia, rice, and *Arabidopsis* (Hamiaux et al., 2012; Kagiyama et al., 2013; Zhao et al., 2013). In addition, a judicious labeling on the D-ring, the key element for the bioactivity of SLs (Boyer et al., 2012), would be useful in the understanding of the mode of action for this class of compounds as a plant hormone.

METHODS

Synthesis: General Remarks

Infrared spectra were recorded neat. ^{1}H and ^{13}C NMR spectra were recorded as solutions in $CDCl_3$, using residual protic

solvent $CHCl_3$ (δ_H = 7.24 ppm) or $CDCl_3$ (δ_C = 77.23 ppm) as an internal reference. For the ^1H spectra, data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet), coupling constant in Hz, integration. Mass spectra were determined by electrospray ionization (ESI). IR spectra were reported in reciprocal centimeters (cm^{-1}). All reactions were monitored by thin layer chromatography (TLC) carried out on 0.2 mm aluminum silica gel pre-coated plates using UV light and 5% ethanolic solution of phosphomolybdic acid and heat as staining agent. Flash chromatography was performed on 40–63 µm (400–230 mesh) silica gel 60 with ethyl acetate (EtOAc)-heptane as eluents. Commercially available reagents and solvents were purified and dried when necessary by usual methods. Unless otherwise mentioned, all other reagents were purchased from commercial sources and were used without further purification.

SL analogs GR24, **23**, and **31** were supplied as described by Boyer et al. (2012), CISA-1 by Rasmussen et al. (2013), and 4BD by Fukui et al. (2011). SL analogs AR8, AR32, and AR36 were prepared as below: 5-chloro-3,4-dimethylfuran-2(5*H*)-one and methyl malondialdehyde sodium salt were synthesized according to the literature procedures (Canevet and Graff, 1978; Nair et al., 1981).

Aldehyde AR8

Methyl malondialdehyde sodium salt (244 mg, 2.6 mmol) was dissolved in DMSO (2 ml). 5-chloro-3,4-dimethylfuran-2(5H)-one (165.5 mg, 1.13 mmol) was added and the mixture stirred at room temperature for 14 h. The reaction was checked for completion by TLC (heptane/EtOAc, 8:2, v/v) and water was added until the entire dissolution of salts. Organic materials were extracted with CH_2Cl_2 three times and the combined organic layers dried over $MgSO_4$, filtrated, and evaporated to dryness. The resulting residue was purified by chromatography on a silica gel column with a linear gradient of EtOAc (0–50%) in heptane as the mobile phase, giving aldehyde AR8 as a white powder (202 mg, 1.03 mmol, yield = 91%). R_f = 0.13 (heptane/EtOAc, 8:2, v/v), ^1H NMR (500 MHz, $CDCl_3$) δ: 9.36 (s, 1H), 7.18 (s, 1H), 6.01 (s, 1H), 2.08 (s, 3H), 1.94 (s, 3H), 1.73 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 191.5, 170.9, 161.4, 152.6, 128.2, 123.3, 101.8, 11.4, 8.8, 6.6. IR υ_{max} (film, cm^{-1}): 1771, 1684, 1652, 1168, 978. HRMS (ESI, positive mode): *m/z* = 197.0809 $[M+H]^+$, calcd for $C_{10}H_{13}O_4$ = 197.0814.

(2*E*,4*E*)-Methyl 5-((3,4-Dimethyl-5-Oxo-2,5-Dihydrofuran-2-Yl)Oxy)-4-Methylpenta-2,4-Dienoate AR36

Aldehyde AR8 (100 mg, 0.5 mmol) was dissolved in dry toluene (10 ml), commercially available methyl (triphenylphosphoranylidene)acetate (203 mg, 0.61 mmol) was added, and the resulting mixture was stirred at reflux for 12 h. The reaction was checked for completion by TLC (heptane/EtOAc, 7:3, v/v). The reaction mixture was concentrated under reduced pressure and the resulting residue purified by chromatography on a silica gel column with a linear gradient of EtOAc (0–20%) in heptane as mobile phase, giving the target dienoate AR36 as a white powder (77 mg, 305 µmol, yield = 61% (80% after recovery of unreacted starting aldehyde AR8)). ^1H NMR (300 MHz, $CDCl_3$) (Supplemental Figure 6A) δ: 7.24 (d, *J* = 15.4 Hz, 1H), 6.73 (s, 1H), 5.89 (s, 1H), 5.82 (d, *J* = 15.4 Hz, 1H), 3.75 (s, 3H), 2.02 (s, 3H), 1.89 (s, 3H), 1.76 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) (Supplemental Figure 6B) δ: 171.2, 167.7, 153.0, 148.3, 144.9, 127.8, 117.4, 115.1, 101.6, 51.5, 11.4, 9.4, 8.6. IR υ_{max} (film, cm^{-1}): 1767, 1718, 1639, 1317, 1154, 979. HRMS (ESI, positive mode): *m/z* = 253.1076 $[M+H]^+$, calcd for $C_{13}H_{17}O_5$ = 253.1076.

(*E*)-3,4-Dimethyl-5-((2-Methylbuta-1,3-Dien-1-Yl)Oxy) Furan-2(5*H*)-One AR32

Methyl triphenylphosphonium bromide (1.25 g, 3.5 mmol) was dissolved in dry THF (4 ml) and cooled to –50°C. Butyllithium (2.25 ml, 3.6 mmol, 1.6 M in hexane) was added dropwise and the resulting mixture was warmed to –10°C for 45 min, after which it was cooled to –78°C. A solution of AR8 (400 mg, 2.04 mmol) in THF was added dropwise to the preceding ylide and stirred for 2 h 30 min at –50°C. The reaction mixture was then allowed to warm to –20°C before being poured in the CH_2Cl_2-phosphate buffer (pH 7) mixture (v/v; 1/1). The mixture was extracted with CH_2Cl_2, the organic layer was washed with water, dried under reduced pressure, and the resulting residue purified by chromatography on a silica gel column with a linear gradient of EtOAc (0–50%) in heptane as mobile phase, giving the target diene AR32 as a slightly yellow oil (150 mg, 773 µmol, yield = 38%). ^1H NMR (500 MHz, $CDCl_3$) δ: 6.41 (s, 1H), 6.22 (dd, *J* = 17.2, 10.7 Hz, 1H), 5.77 (s, 1H), 5.08 (d, *J* = 17.2 Hz, 1H), 4.94 (d, *J* = 10.7 Hz, 1H), 1.98 (s, 3H), 1.85 (s, 3H), 1.71 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.6, 154.2, 142.4, 135.5, 127.4, 119.2, 111.0, 101.8, 11.4, 9.04, 8.4. IR υ_{max} (film, cm^{-1}): 1766, 1150, 960. HRMS (ESI, positive mode): *m/z* = 195.1020 $[M+H]^+$, calcd for $C_{11}H_{15}O_3$ = 195.1021.

Chemical Stability

GR24, **23**, **31**, and AR36 were tested for their chemical stability in an aqueous solution. Aqueous solutions of the compound to be tested (50 µg ml^{-1}) were incubated at 21°C in the HPLC vials. The compounds were first dissolved in acetone (1 ml). Then, 50 µl of the previous solutions were diluted to the final concentration with ethanol (175 µl) and water (750 µl). Indanol (25 µl of a 1 mg ml^{-1} solution in acetone) as internal standard was added to each solution. The time course of degradation was monitored by UPLC analysis using an Acquity UPLC HSS C_{18} column (1.8 µm, 2.1 × 50 mm), eluted first by 5% acetonitrile in water containing 0.1% formic acid for 0.5 min then by a gradient from 5% to 100% acetonitrile in water containing 0.1% formic acid within 6.5 min, and by 100% acetonitrile containing 0.1% formic acid for 3 min. The column was operated at 40°C with a flow rate of 0.6 ml min^{-1}. Compounds eluted from the column were detected with a

photodiode array detector. The relative quantity of remaining (non-degraded) product was determined by integration comparison with the internal standard.

Plant Assays

Bud Outgrowth Inhibition Activity Assay

Pea *rms1* mutant plants (allele *rms1-10* identified in the dwarf line cv. Térèse) (Beveridge et al., 1997; Rameau et al., 1997; Sorefan et al., 2003; Gomez-Roldan et al., 2008) deficient in SLs were used for the bioassay. Pea *rms4* mutant plants (allele *rms4-3* identified in the dwarf line Térèse) (Johnson et al., 2006) not responding to SLs were used for the bioassay. The compounds to be tested were applied directly to the bud with a micropipette as 10 µl of solution containing 0.1% acetone with 2% polyethylene glycol 1450, 50% ethanol, and 0.4% DMSO. The control 0 is the treatment with 0.1% acetone. Twenty-four plants were sown per treatment in trays (two repetitions of 12 plants). The treatment was generally done 10 d after sowing, on the axillary bud at node 4 (or 3) (Boyer et al., 2012). The branches at nodes 1 to 2 were removed to encourage the outgrowth of axillary buds at nodes above. Nodes were numbered acropetally from the first-scale leaf as node 1 and cotyledonary node as node 0. Bud growth at node 4 (node 3) was measured 8–10 d after treatment with digital calipers. Generally, if poor activity or no activity was found at 1-µM concentration, no replication was done.

Pea Hydroponic Culture

The high branching SL-deficient pea *rms1* mutant plants (*rms1-10*) were used. The nutrient solution (100%) was prepared by adding, in water (1000 l), the following macronutrients: HNO_3 (0.28 L), $(NH_4)_2HPO_4$ (120 g), $Ca(NO_3)_2$ (40 g), $Mg(NO_3)_2$ (140 g), KNO_3 (550 g), $(NH_4)_2MoO_4$ (0.05 g), H_3BO_3 (15 g), $MnSO_4.H_2O$ (2 g), $ZnSO_4.7\ H_2O$ (1 g), $CuSO_4.5\ H_2O$ (0.25 g), Sequestrene® (10 g) (Fe-EDTA solution). Pea seeds were germinated in wet sand for 6 d. Germinated seeds were placed in premade holes in the lid (35 holes per lid, 20-mm diameter) of a hydroponic PVC opaque pot containing the hydroponic culture solution (47 L, pH 5.8). Acetone or compound to be tested (dissolved in acetone) was added to the hydroponic culture solution to give a final concentration of 0, 1, or 3 µM of compound to be tested and 0.01% acetone. The hydroponic culture solution was continuously aerated by an aquarium pump and was replaced weekly. Eight days after the beginning of the treatment, branch/lateral bud lengths (nodes 1 to 6) were measured with an electronic caliper.

Gene Expression Analysis

PsBRC1 Transcript Levels

The different compounds were applied directly to the bud as done in branching bioassays. The axillary buds at node 3 from 30 plants per treatment and biological repeat were dissected. Axillary buds were harvested, total RNA isolated and quantified, cDNA synthesized, and real-time PCR gene expression analyses conducted as described by Braun et al. (2012). Quantitative real-time PCR analyses were performed using SYBR ROX RealMasterMix (5Prime). Cycling conditions for amplification were 95°C for 10 min, 50 cycles of 95°C for 5 s, 62°C for 5 s, and 72°C for 15 s followed by 0.1°C s^{-1} ramping up to 95°C for fusion curve characterization. Three biological repeats were analyzed in duplicate. To calculate relative transcript levels, the comparative cycle method based on non-equal efficiencies was used (Kgosi et al., 2012). Transcript levels for the different genes were expressed relative to the expression of *EF1*α gene (Johnson et al., 2006). Primer sequences were as follows: PsBRC1 forward (5′-TCGAAAGACGG AATCAAACA-3′) and PsBRC1 reverse (5′-TCCCTTGCTCTTTC TCTTGC-3′); EF1α forward (5′-TGTGCCA GTGGGACGTGTTG-3′) and EF1α reverse (5′-CTCGTGGTGCATCTCAACGG-3′).

Adventitious Rooting Assay

Induction and measurement of adventitious roots in intact *Arabidopsis* plants were performed as previously described (Rasmussen et al., 2012, 2013), with minor modifications. *Arabidopsis* seeds (wild-type Columbia (Col-0) and mutant homozygous seed lines: *max2-1*, *max4-1* were gas sterilized for 4 h and sown on square agar plates containing 10 g L^{-1} phytagel (Sigma-Aldrich, www.sigmaaldrich.com); 5 g L^{-1} sucrose; 1.5 g L^{-1} Murashige and Skoog (MS) salts (Phytotechnology Laboratories, www.phytotechlab.com); 0.5 g L^{-1} MES. SL analogs were dissolved in acetone, diluted with water, and then treatments were made up in water. In the response comparison of all *max* mutants, 1 µM of SL analog was used while in the dose-response experiment concentrations used were 0, 1 nM, 10 nM, 100 nM, and 1 µM, and the treatments were added to the agar media prior to pouring the plates. Plates were then stratified in the dark at 4°C for 3 d then exposed to 8 h of light before etiolation for 4 d. After etiolation, *Arabidopsis* seedlings were grown in 16:8 day:night conditions at 22°C. The number of adventitious roots on each seedling was then counted 7 d after removal from etiolation conditions. Adventitious roots were only counted above the root–shoot junction (and not including any roots that formed at the junction). Wild-type and *max4* dose-response experiments were done 24 h apart for **31**, AR36, or GR24 and together for **23** and GR24.

Lateral Root Density Assay

For lateral root (LR) density measurements, lateral roots were counted on seedlings grown on media (1/2 MS) containing 1 µM-concentration of each of the analogs. The number of LRs was determined under the stereomicroscope (Leica, S4E) and IMAGEJ was used to quantify the root lengths. The LR density was then calculated as the number of LRs per cm

of the primary root. At least three independent biological repeats were performed with at least 30 plants per repeat and per condition. Statistical analyses were performed using a mixed-model ANOVA analysis via SAS.

Germination Stimulation Activity Assay

Two populations of *P. ramosa* were used in this study: pathovar C and T (Benharrat et al., 2005). Seeds of *P. ramosa* pathovars C (pv C) were collected from Saint Martin de Fraigneau, France, on broomrape parasitizing winter oilseed rape (*Brassica napus* L.) in 2005 and seeds of *P. ramosa* pathovars T (pv T) from Sarthe, France, on broomrape developed on hemp (*Cannabis sativa* L.) in 2007. Seeds of *Orobanche cumana* Wallr. were collected from broomrape parasitizing sunflower, *Helianthus annuus* L., Longeville-sur-mer, France, in 2009; seeds of clover broomrape (*Orobanche minor* Sm.) from parasite red clover (*Trifolium pratense* L.) were kindly provided by Pr K. Yoneyama (Japan) and seeds of *S. hermonthica* (Del.) Benth were collected from Gadarif, East Sudan, in 1999. The seeds were stored dry in the dark at 25°C.

GR24 and SL analogs were re-suspended in acetone at 10 mmol L^{-1}, then diluted with water at 1 mmol L^{-1} (water/acetone; v/v; 99/1). Dilutions of 1×10^{-3} mol L^{-1} to 1×10^{-15} mol L^{-1} are then performed in water/acetone (v/v; 99/1). Seeds of parasitic plants were surface-sterilized according to Dos Santos et al. (2003), then re-suspended in sterile water (10 g L^{-1}) and distributed in 96-well plates (50 µl ≈ 100 seeds per well). After a conditioning period (7 d, 21°C, in dark, sealed plate, except for *S. hermonthica* seeds that were conditioned at 30°C), GR24 (10×) or other molecules were added and volumes were adjusted to 100 µl with water (water/acetone; v/v; 999/1). In plate, a range of concentrations from 10^{-13} to 10^{-6} mol L^{-1} were applied for GR24, **31**, and **23,** and from 10^{-12} to 10^{-5} mol L^{-1} for AR36. Controls were made with water/acetone (v/v; 999/1) and without seeds. Plates were incubated for germination (21°C, in the dark or 30°C for *S. hermonthica*.). After 4 d, the methylthiazolyldiphenyl-tetrazolium bromide assays, as high-throughput seed germination assay recently described (Pouvreau et al., 2013), were carried out according to Mosmann (1983) with minor modification of the solubilization buffer (Triton X-100 10%, HCl 0.04 mol L^{-1} in isopropanol). Each germination assay was repeated at least three times. For each compound tested, dose–response curves (A = f(c), A: Absorbance 570 nm; c: concentration (mol L^{-1})) and half maximal effective concentration EC_{50} and maximum of germination percentage were determined using a Four Parameter Logistic Curve computed with SigmaPlot® 10.0.

Gigaspora rosea Germ Tube Branching Bioassay

Spores of *G. rosea* (DAOM 194757) produced in pot culture of leek were collected by wet sieving and washed as already described (Besserer et al., 2006). Branching bioassays were performed on M media (Becard and Fortin, 1988) supplemented with 10 µM quercetin (SIGMA) and 0.5% Phytagel (SIGMA), following the protocol established by Buee and co-workers (2000). Briefly, spores were germinated in two-compartment Petri dishes incubated vertically under 2% CO_2 and 30°C, allowing germ tubes to grow upwards. Six days after inoculation, small wells were made with the tip of a Pasteur pipette at a distance of 5 mm of the tip of each germ tube; 5 µl of the tested solution were then applied in each well. Spore producing small or double germ tubes were discarded from the analysis. Newly formed apices were counted 48 h after molecule application. All tested molecules were dissolved in acetonitrile/water 10% (v:v) at a final concentration of 10^{-7} M. Acetonitrile 10% and GR24 (10^{-7} M) were used as negative and positive controls, respectively. Mean values were compared using the Student's *t*-test ($P < 0.05$). Twenty to 30 spores were used for each treatment.

Statistical Analyses

Because deviations from normality were observed for axillary bud length after SL treatment, the Kruskal–Wallis test was used to assess the significance of one treatment with one analog in comparison to treatment with another using R Commander version 1.7–3 (Fox, 2005). In other cases, one-way ANOVA or Student's *t*-tests were used.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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