RUNNING TITLE: Strigolactone-gibberellin interaction in apical dominance

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TITLE: Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*)

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Abstract

The role of strigolactones as plant growth regulators has been demonstrated through research on biosynthesis and signaling mutant plants and through the use of GR24, a synthetic analog of this class of molecules. Strigolactone mutants show a bushy phenotype and GR24 application inhibits the growth of axillary buds in these mutants, thus restoring the phenotype of a wild plant, which is characterized by a stronger apical dominance. In this work, we tested the effectiveness of this chemical on pea (*Pisum sativum*) plants following apex removal, which disrupts apical dominance and leads to axillary bud outgrowth. Moreover, we searched for relationships between the response to the strigolactone and gibberellin metabolism by applying GR24 to both climbing and dwarf peas, the latters being mutants for gibberellin biosynthesis. The results suggest that the endogenous level of the bioactive gibberellin GA_1 might modulate the response of decapitated pea plants to GR24, by changing bud sensitivity to the applied strigolactone.

Keywords: apical-dominance; bud; gibberellin; pea; strigolactone

Abbreviations: AD = apical dominance

fw = fresh weight

Introduction

Apical dominance is the control exerted by the shoot apex over lateral bud outgrowth. If the shoot apex is decapitated, AD is released and one or more lateral (axillary) buds begin to grow out (Cline, 1997). This developmental control may deeply influence the architecture of the plant and ultimately the ecological fitness of the whole organism (Aarssen, 1995). For this reason and for the numerous consequences on agricultural techniques, AD has been extensively studied for many years. Plant growth regulators play a pivotal role in the control of AD and several models have been proposed to explain their function. Since Thimann and Skoog's pioneering experiments (1933; 1934), auxin is considered as a primary signal for axillary bud inhibition, although its precise mechanism of action is not yet clear. The removal of the shoot apex disrupts or weakens AD, by discontinuing the basipetal flow of indole-3-acetic acid (IAA) which originates from the apex itself (Ongaro and Leyser, 2008). Despite this evidence, it has been demonstrated that the polarly transported IAA does not need to enter the bud to exert its inhibitory action (Booker et al., 2003), therefore the existence of a secondary messenger, mediating the auxin signal, had been anticipated (Li et al., 1995). Recent studies on a series of bushy mutants of several species (*Petunia hybrida*, *Oryza sativa*, *Arabidopsis thaliana* and *Pisum sativum*) led to the identification of a good candidate for this role of secondary messenger: the strigolactones, a group of terpene lactones previously known as biochemical signals released by the root for the attraction of symbiotic fungi, as well as germination stimulants for parasitic plant seeds (Beveridge et al., 1994; 1997; Booker et al., 2004; 2005; Ongaro and Leyser, 2008). The bushy phenotype of the aforesaid mutants is caused by defects in the biosynthesis or signal perception/transduction of strigolactones, therefore these molecules, or their derivatives, are now classified as plant growth regulators (Gomez-Roldan et al., 2008; Umehara et al., 2008). The use of GR24, a synthetic analog of strigolactones, has been decisive to demonstrate the inhibitory effect of these molecules on axillary bud outgrowth. More

informations might be obtained by testing the effectiveness of GR24 on different systems, like, for instance, decapitated plants with a normal genotype for strigolactones. For this reason, we investigated the effects of GR24 application, at different concentrations, to the buds of decapitated pea (*Pisum sativum*) plants. Our work also aimed at studying some aspect of the cross-talking between strigolactones and other classes of plant growth regulators. Among the latters, gibberellins represent a group whose involvement in the control of AD may have been overlooked. There is a general lack of data on this subject. Early evidence suggest that gibberellins might participate to the network of hormonal signals underlying AD. For example, gibberellins enhance the auxin-induced inhibition of lateral bud outgrowth in decapitated pea plants (Jacobs and Case, 1965; Scott et al., 1967) and treatments with gibberellin biosynthesis inhibitors may lead to loss of AD in some species (Ruddat and Pharis, 1966). In recent years, the involvement of gibberellins in AD has received little attention and very few papers have been published on this subject. Jiang et al. (2010) have demonstrated that transgenic Chrysanthemum plants, transformed with the *lateral suppressor*-like gene in both the sense and antisense orientation, branched more profusely than the wild-type or did not branch, respectively. The bushy phenotype (sense transformants) was associated with a reduced gibberellin content, while the antisense (non branching) transformants had higher gibberellin levels. A citrus hybrid rootstock overexpressing antisense *CcGA20ox1*, a key enzyme of gibberellin biosynthesis, showed lower levels of bioactive GA_1 compared to the control and this correlated with a bushy phenotype (Fagoaga et al., 2007). Our work started from the evaluation of the response of decapitated pea plants to GR24 application. Furthermore, our interest was also to investigate how this response may be affected by gibberellin metabolism: to address this problem, we carried out the experiments on both a climbing cultivar and a dwarf one, the latter being a mutant defective in gibberellin biosynthesis, as we assessed through our analyses.

Materials and methods

Plant growth

Pea (*Pisum sativum* L.) plants belonged to two commercial cultivars: the climbing 'Lavagna' and the dwarf 'Meraviglia d'Italia' (Gargini Sementi, Italy). The experiments were performed in a glasshouse in Pisa (Italy; 43° 43' N, 10° 24' E, sea level). Plants were grown under natural sunlight, between late April and late May 2010, with average temperatures ranging from 20 to 25°C during the day and from 12 to 18°C at night. Seeds were imbibed under running tap water for 6 h and surface sterilized by dipping them in 70% ethanol for 2 min, then in 2% sodium hypochlorite for 30 s. They were rinsed with abundant sterile distilled water and left to germinate in 10 L glass vessels with sterile vermiculite, wetted with 500 mL sterile distilled water, in darkness at 25°C for 36 h. Germinated seeds were transplanted in 0.2 L pots in sterile vermiculite and irrigated with 50 mL 0.3x Hoagland solution no. 2 (Sigma, Italy). Plants were then watered every 2 d, alternating 40 mL sterile distilled water to an equal volume of Hoagland solution 0.3x.

Treatments with the strigolactone

Pea plants with 5 fully developed internodes (*i.e.* 10 d after transplanting in pot) were decapitated by a scalpel just above the fourth node. The resting buds at this node were treated with solutions of GR24 (Chiralix, The Netherlands), a synthetic analog of strigolactones, at 3 concentrations: 0.05, 0.1 and 1 µM. Ten µL of each solution were applied to the buds by a microsyringe. To prepare the solutions, GR24 was first dissolved in a small volume of acetone, then diluted to the final concentration with sterile distilled water. The final acetone concentration was 0.02%. The buds of the fourth node of control plants were treated with an equal volume of 0.02% acetone in sterile distilled water. The elongation of the treated buds was measured daily by a caliper. The greatest

difference between the mean length of treated and control buds was recorded 6 d after GR24 application, therefore we used such data to evaluate the effect of the treatments. All experiments were carried out in duplicate and each replication of each thesis was made of 30-35 plants. Data from the two replications were averaged for each thesis, because they did not differ statistically (consequently, n = 60-70 for each thesis).

Statistical analysis

GR24 treated plants and the related control were compared for the elongation of the bud of the fourth node by one-way ANOVA. Analysis was performed separately on climbing and dwarf plants. Means were discriminated using Newman-Keuls test.

Biochemical characterization of the cultivars

To characterize the biochemical phenotype of the two cultivars, the concentration of the main endogenous gibberellins was determined in stem segments made by both nodal and internodal tissues. Samples (approximately 0.5 g each) were taken from 10 d old plants, by cutting with a scalpel a stem segment which included the fourth node and the 5 mm of internodal tissue above and below the node. Samples were analyzed for the concentration of the bioactive GA_1, its biosynthesis precursor GA_{20} and its direct catabolite GA_8. Briefly, samples were supplemented with suitable amounts of deuterated GA_1, GA_{20} and GA_8, extracted three times with 80% methanol with 0.05% acetic acid and the pooled extracts were purified by HPLC: the column was a Thermo Hypersil C18, 150 x 4.5 mm ID (Thermo, USA), eluted at a flow of 1 mL min^{-1} with methanol in 0.05% acetic acid (2 min 10% methanol, then a linear gradient from 10% to 100% methanol in 20 min). Fractions corresponding to the elution volume of the standard gibberellins were collected, then

dried and silylated with bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Pierce, USA). Gas chromatography-mass spectrometry analysis was performed on a Saturn 2200 mass spectrometer coupled to a CP-3800 gas chromatograph (Varian, USA) equipped with a MEGA 1 capillary column (MEGA, Italy) 25 m x 0.25 mm ID x 0.25 μm film thickness, coated with 100% dimethylpolysiloxane. Details of the GC-MS analysis were given in Fambrini et al. (2011). The concentration of gibberellins were calculated by averaging the data from three biological replicates (for each of them $n = 15$) for each cultivar.

Results and discussion

Gibberellin metabolism

The content of bioactive gibberellins was assessed in both cultivars, by analyzing shoot sections. The level of the main bioactive gibberellin (GA_1) was nearly 2.6 times lower in the dwarf cultivar than in the climbing one, while there were not significant differences either for the immediate metabolic precursor of GA_1 (*i.e.* GA_{20}), or its catabolite (GA_8; Fig. 1). These data confirmed that the dwarf cultivar "Meraviglia d'Italia" had a reduced GA 3-oxidase activity, plausibly owing to a mutation affecting one or more *PsGA3ox* genes. This type of mutation is known to reduce stem length in many widely used commercial cultivars of pea (Atwell et al., 2003). Our data demonstrate that the dwarf cultivar, with its reduced content of GA_1, has an altered gibberellin metabolism. The defect resides in the activation of GA_{20} to GA_1, which makes such cultivar suitable for our investigation on the interaction between strigolactones and gibberellins, through the comparison with the climbing genotype.

Response to GR24

The plants of both cultivars showed a strong degree of AD. This was assessed by growing some plants over a period of several weeks, well exceeding the length of our experiments. When left to grow undisturbed, under the conditions set up for the experiments, these test plants showed a characteristic morphology of the aerial part, *i.e.* a single stem without branches, regardless to the genotype. The chosen experimental conditions did not induce, in these intact plants, the outgrowth of the axillary buds, that were barely visible and reached an average length of about 0.5 mm (data not shown). Decapitation of the plant above the fourth node always caused the outgrowth of one axillary bud at the node just below the cut and also at the lower nodes, in both cultivars. The concentrations of GR24 that were applied in our experiments were chosen on the basis of a previous work on pea, in which the axillary buds were treated with 0.1 µM GR24 (Gomez-Roldan et al., 2008). The effects of, respectively, lower and greater doses (*i.e.* 0.05 and 1 µM) were also tested in the present work. A single application of GR24 to the axillary buds of the fourth node following decapitation induced different responses, expressed as elongation of the treated bud 6 d after the treatment. The response was dependent on the concentration of the chemical and on plant genotype. The elongation of the treated bud of plants of the climbing cultivar decreased with increasing GR24 concentration (Fig. 2A), reaching 74%, 66% and 46% of the corresponding control with 0.05, 0.1 and 1 µM, respectively. This confirmed the effectiveness of the molecule in the inhibition of bud outgrowth consequent to the disruption of AD. The response of the dwarf cultivar to GR24 followed an opposite pattern: increasing the concentration of the molecule led to progressively more elongated buds. These were 41%, 64% and 84% of the corresponding control with 0.05, 0.1 and 1 µM, respectively (Fig. 2B). Furthermore, despite the average length of the buds treated with 1 µM GR24 was lower than the control, such difference was not statistically significant ($P > 0.05$). The application of increasing amounts of GR24 in this range of concentrations apparently led to a progressive loss of efficacy of the molecule for the dwarf plants, while for the climbing ones the

inhibition of bud outgrowth was proportional to the dose administered. The results of the present work suggest that gibberellins may be involved in the control of AD. Although every interpretation of these data will be speculative, they may provide the basis for a discussion. The pattern of the dose-response relationship for strigolactone inhibition of bud growth is not known. Nevertheless, dose-response curves have been reported for strigolactone stimulation of germination of parasitic plant seeds. These curves, relating to both natural (sorgolactone) and synthetic (GR24) strigolactones applied to the seeds of several parasitic species, show either a sigmoidal or a bell shape, depending also on the dose administered and the plant species. Sigmoidal-shaped curves display a suboptimal (linear) zone at low strigolactone concentrations, reaching an optimal (saturation) zone at high concentrations. Bell-shaped curves show also a supraoptimal zone, characterized by a decline of the response at considerably high strigolactone concentrations (Wigchert et al., 1999). This pattern could hold true also for GR24 inhibition of axillary buds. In pea plants, gibberellins seem to modulate the response to the applied strigolactone through changes in the sensitivity of the bud. In this view, relatively high levels of the bioactive GA_1 would lower bud sensitivity to GR24, as in climbing pea plants, thus keeping the applied concentrations within a range which is suboptimal, *i.e.* in the linear part of the hypothetical dose-response curve of the strigolactone. The result would be a stronger inhibition of bud outgrowth with increasing amounts of the strigolactone. Conversely, the low levels of GA_1 of the dwarf plants would enhance the sensitivity of the buds to GR24, thus shifting the response towards the supraoptimal (declining) zone of the hypothetical dose-response curve of the strigolactone. Increasing the concentration of GR24 would elicit weaker responses in dwarf pea plants, whose bud elongation is progressively less inhibited until, at the greater dose applied, GR24 loses its effectiveness. Much work is needed to advance our understanding of the hypothesized gibberellin-strigolactone interaction, which may represent one of the nodal points of the complex hormonal network underlying the control of AD. Such nodal point will have to be identified and properly placed within a model outlining the

network and this will require extensive studies. The nature of the relationship between gibberellin and strigolactone signals must be investigated in depth: its complexity is totally unknown and it may be expected that further hormonal signals impinge on it. For instance, it is known that apex-derived auxin induces gibberellin biosynthesis in the stem of pea (Ross et al., 2000). Based on this evidence, it could be inferred that the role of gibberellin in the control of AD would be, at least partly, that of a second messenger of auxin. It is likely that auxin action may be modulated through the changes that it may induce in gibberellin biosynthesis pattern, nevertheless it cannot be excluded that gibberellins and strigolactones may interact also independently of auxin. Further experiments with GR24 treatments at concentrations both lower and greater than those applied in the present work will help to outline the dose-response relationship between strigolactones and bud inhibition. Moreover, analyses of the concentrations of endogenous plant growth regulators, along with the study of the related biosynthesis and signal transduction genes, are required to highlight the dynamics of these signal molecules in GR24-treated tissues.

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Legends of figures

Fig. 1. Concentration of the main endogenous gibberellins in stem sections of 10 d old pea plants. Empty bars: cv. 'Lavagna'. Filled bars: cv. 'Meraviglia d'Italia'. Data are the mean of three biological replicates ± SE (n = 15 for each replicate).

Fig. 2. Bud elongation of decapitated pea plants, 6 d after the treatment with GR24 at different concentrations. Bars represent the mean of two independent replicates (n = 60-70) ± SE. Different letters indicate statistically significant differences according to Newman-Keuls test ($P < 0.05$). (A) Cultivar 'Lavagna' (climbing). (B) Cultivar 'Meraviglia d'Italia' (dwarf).

Fig. 1

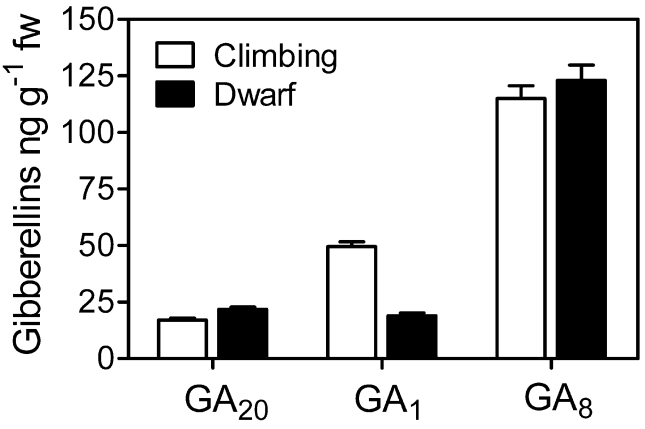

Fig. 2