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Strigolactones in the *Rhizobium*-legume symbiosis: Stimulatory effect on bacterial surface motility and down-regulation of their levels in nodulated plants

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ABSTRACT

Strigolactones (SLs) are multifunctional molecules acting as modulators of plant responses under nutrient deficient conditions. One of the roles of SLs is to promote beneficial association with arbuscular mycorrhizal (AM) fungi belowground under such stress conditions, mainly phosphorus shortage. Recently, a role of SLs in the *Rhizobium*-legume symbiosis has been also described. While SLs' function in AM symbiosis is well established, their role in the *Rhizobium*-legume interaction is still emerging. Recently, SLs have been suggested to stimulate surface motility of rhizobia, opening the possibility that they could also act as molecular cues. The possible effect of SLs in the motility in the alfalfa symbiont *Sinorhizobium meliloti* was investigated, showing that the synthetic SL analogue GR24 stimulates swarming motility in *S. meliloti* in a dose-dependent manner. On the other hand, it is known that SL production is regulated by nutrient deficient conditions and by AM symbiosis. Using the model alfalfa-*S. meliloti*, the impact of phosphorus and nitrogen deficiency, as well as of nodulation on SL production was also assessed. The results showed that phosphorus starvation promoted SL biosynthesis, which was abolished by nitrogen deficiency. In addition, a negative effect of nodulation on SL levels was detected, suggesting a conserved mechanism of SL regulation upon symbiosis establishment.

1. Introduction

Strigolactones (SLs) are secondary metabolites classified since 2008 as a new class of plant hormones regulating several different processes in plants [1,2]. They modulate the development of both roots and shoots in response to nutrient deficient conditions. Accordingly, SLs regulate several processes in plant physiology such as above- and belowground plant architecture, adventitious rooting, secondary growth, leaf senescence and reproductive development [1–8]. In addition, new roles for SLs, including the response to abiotic stresses [9–13] and defence responses [14,15], are being continuously discovered. Despite their recent functions as phytohormones, they were firstly identified as signalling molecules in the rhizosphere, indicating their biological and ecological relevance. There, they function as seed germination stimulants for root parasitic plants of the family Orobanchaceae, including the genera *Striga*, *Orobanche* and *Phelipanche*, and as host detection signals for symbiotic arbuscular mycorrhizal (AM) fungi from the phylum Glomeromycota [16–18]. In the case of AM symbiosis, SLs act as fungal hyphal branching factors and spore germination stimulants during the pre-symbiotic stage, thus facilitating plant host-AM fungi interaction [16,19]. Accordingly to SLs' role as molecular cues in the rhizosphere under nutrient deficient conditions, together with other molecules [20,21], they are mainly produced in the roots and their biosynthesis is promoted by these unfavourable conditions, mainly phosphorus (P) and nitrogen (N) deprivation [22–24]. Biosynthetically, SLs are derived from the carotenoids [22,25], thus belonging to the apocarotenoid class [26]. They are produced and secreted at extremely low levels – pico and nanomolar –, which hampers their analysis and quantification [27]. It is well known that each plant produces a blend of different SLs which depends on the species [27], although it has been proposed that a given plant would only produce SLs from the same stereochemical family. Albeit with some differences, all natural SLs show a similar chemical structure

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consisting of a structural core formed by a tricyclic lactone (the ABC-rings) connected via an enol ether bridge to a butyrolactone group (the D-ring) (Fig. S1) [27,28]. In addition to the canonical SLs, the existence of other non-canonical SLs has been recently shown in Arabidopsis and rice [29,30]. This compound, identified as methyl carlactonate, was shown to inhibit shoot branching, a phenotype associated with SLs [29].

More recently, a role for SLs in another important beneficial plant-symbiotic microorganism interaction that takes place in the rhizosphere – the *Rhizobium*-legume symbiosis – has been shown [31–33]. However, their specific role in this symbiosis is so far unknown. Through this association, which dates back more than 60 million years, diverse species of soil bacteria, collectively known as rhizobia, are able to invade legume roots in nitrogen-limiting environments, leading to the formation of the root nodules [34,35]. The nodule is a highly specialized organ where endosymbiotic rhizobia reduce atmospheric nitrogen into ammonia, which is used by the plant improving plant nutrition and growth. In return, the bacteria are supplied with carbohydrates in a protected environment. The formation of symbiotic N_2-fixing nodules is a complex process as it requires two different developmental processes: bacterial infection initiated in the epidermis and nodule organogenesis that takes place in the root cortex. These two processes need to be coordinated in time and space, and require a continuous and adequate signal exchange between the two partners [34,35]. This chemical communication begins with the production and exudation into the rhizosphere of specific flavonoids by the host plant, and whose production is increased under nitrogen-limiting conditions [36]. These flavonoids act as chemoattractants for specific bacteria and as inducers of the expression of *nod* genes which are involved in the biosynthesis of the Nod factors, lipo-chitooligosaccharides required for nodule formation on the host root [37,38]. Nod factors, together with additional microbial signals such as polysaccharides and secreted proteins, allow the rhizobia attached to root hairs to penetrate the root through infection threads and reach the nodule primordium, where bacteria are released into the plant cytoplasm to finally differentiate into N_2-fixing bacteroids [34,35]. The first evidence for the involvement of SLs on the establishment of the *Rhizobium*-legume symbiosis was obtained in a study in which application of the synthetic strigolactone analogue GR24 increased nodule formation in alfalfa plants inoculated with *Sinorhizobium meliloti* [33]. This positive effect on nodulation, however, was not linked to a stimulatory effect of GR24 on the bacterial growth or the expression of *nod* genes. Studies performed with legume plants with altered endogenous SL levels clearly demonstrated that these phytohormones are not essential for the development of a functional nodule, but may instead be required for the development of optimal nodule number [32,39]. Foo and Davis showed that the pea SL-deficient mutant *rms1* established about 40% less nodules than the corresponding wild-type, phenotype that was partially rescued by exogenous GR24 application [32]. Similarly, Liu et al. showed that *Lotus japonicus LjCCD7*-silenced plants, with an about 80% reduction in SLs, carried 20% less nodules than control plants [39]. More recently, a dose-dependent effect of GR24 on nodule number has been shown in *Medicago truncatula* [31]. Interestingly, these authors observed a positive or negative effect depending on GR24 concentration. Also in *M. truncatula*, expression of the SL biosynthesis genes *MtD27*, *MtCCD7* and *MtCCD8* has been detected in root hairs, as well as in meristematic cells and in the infection zone of nodules in response to *Rhizobium* and application of Nod factors [40,41]. Combined, these results indicate a role of SLs as an endogenous signal in several stages of the *Rhizobium*-legume interaction.

Although we showed no alteration of bacterial traits in response to GR24 that could explain the positive effect on nodulation [33], a recent finding suggests that SLs may have an additional role in the establishment of the symbiosis by acting as a rhizospheric signal for the bacteria [42]. Tambalo et al. found out that legume seed exudates as well as extracts of the moss *Physcomitrella patens* enhanced swarming motility in *Rhizobium leguminosarum*. Swarming is a specialized type of bacterial translocation that allows the rapid and co-ordinated movement of bacteria across solid surfaces [43,44]. Like the well-known swimming motility, it is dependent on flagellar activity, but while swimming is an individual behaviour that takes place in liquid environments, swarming is a surface-associated multicellular behaviour [43]. Swarming has been extensively studied in pathogenic bacteria due to its close connection with the virulence of these microorganisms. Like other bacterial behaviours that take place on surfaces, swarming leads to dramatic changes in bacterial physiology and gene expression. Swarming motility has also been reported for different rhizobia [45–49]. However, the contribution in the *Rhizobium*-legume symbiosis of swarming or any other type of bacterial surface motility remains elusive. The study by Tambalo et al. revealed that the effects caused by legume seed exudates on bacterial swarming were different, depending on the legume species [42]. The authors extended their findings by showing that crude extracts of a wild-type strain of the moss *P. patens* enhanced swarming motility in *R. leguminosarum*, whereas this promotive effect was reduced when extracts from a SL-deficient strain were used. This led the authors to suggest that SLs may be a signalling molecule able to activate swarming motility in *R. leguminosarum*. Although an indirect effect of SLs in this type of motility could not be ruled out.

It is known that the establishment of mycorrhizal symbiosis down-regulates SL levels in different plants [9,50–53], likely as a mechanism to prevent an excessive colonization that could be costly for the host plant. Whether this is the result of a direct effect of mycorrhization on SL biosynthesis, or an indirect effect through the improvement of the plant nutritional status by the symbiosis is, however, not known. As far as we know, it has not been investigated whether the establishment of the *Rhizobium*-legume symbiosis also affects SL production. In order to gain further insights about the role of SLs in the *Rhizobium*-legume symbiosis, in the present study using the model system alfalfa-*S. meliloti* and the SL analogue 2′-Epi-GR24, we have investigated the possible effect of SLs on bacterial motility. In addition, the impact of nodulation on the production of natural SLs in alfalfa has been assessed.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work and their relevant characteristics are listed in Table 1. *S. meliloti* strains were grown at 30 °C either in complex tryptone yeast (TY) medium [54], in Bromfield medium (BM) (0.04% tryptone, 0.01% yeast extract and 0.01% $CaCl_2{\cdot}2H_2O$) or in minimal medium (MM) containing glutamate (6.5 mM), mannitol (55 mM), mineral salts (1.3 mM K_2HPO_4, 2.2 mM KH_2PO_4 $3H_2O$, 0.6 mM $MgSO_4$ $7H_2O$, 0.34 mM $CaCl_2{\cdot}2H_2O$, 0.022 mM $FeCl_3$ 6H2O, 0.86 mM NaCl), and vitamins (0.2 mg/l biotin, 0.1 mg/l calcium pantothenate). When required, antibiotics were added at the final concentration ($\mu g\,ml^{-1}$) of 200 streptomycin (Sm), 10 tetracycline (Tc) and 75 hygromicin (Hyg) for *S. meliloti*.

The SL analogue 2′-Epi-GR24 was kindly provided by Dr. Xie (Utsunomiya University, Japan). To prepare 2-Epi-GR24, 1 mg of the compound was dissolved in 33 μl pure acetone to obtain a stock solution of 10^{-1} M, which was serially diluted in sterile demiwater to obtain the desired final concentrations. As for controls, the corresponding dilutions in sterile demiwater of the solvent acetone were used.