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Research article

The role of isoflavone metabolism in plant protection depends on the rhizobacterial MAMP that triggers systemic resistance against *Xanthomonas axonopodis* pv. *glycines* in *Glycine max* (L.) Merr. cv. Osumi

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ABSTRACT

Glycine max (L.) Merr. plays a crucial role in both the field of food and the pharmaceutical industry due to their input as plant protein and to the benefits of isoflavones (IF) for health. In addition, IF play a key role in nodulation and plant defense and therefore, an increase in IF would be desirable for better field performance. IF are secondary metabolites and therefore, inducible, so finding effective agents to increase IF contents is interesting. Among these agents, plant growth promoting rhizobacteria (PGPR) have been used to trigger systemic induction of plant's secondary metabolism through their microbe associated molecular patterns (MAMPs) that fit in the plant's receptors to start a systemic response. The aim of this study was to evaluate the ability of 4 PGPR that had a contrasted effect on IF metabolism, to protect plants against biotic stress and to establish the relation between IF profile and the systemic response triggered by the bacteria. Apparently, the response involves a lower sensitivity to ethylene and despite the decrease in effective photosynthesis, growth is only compromised in the case of M84, the most effective in protection. All strains protected soybean against *Xanthomonas axonopodis pv. glycines* (M84 > N5.18 > Aur9>N21.4) and only M84 and N5.18 involved IF. N5.18 stimulated accumulation of IF before pathogen challenge. M84 caused a significant increase on IF only after pathogen challenge and N21.4 caused a significant increase on IF content irrespective of pathogen challenge. Aur9 did not affect IF. These results point out that all 4 strains have MAMPs that trigger defensive metabolism in soybean. Protection induced by N21.4 and Aur9 involves other metabolites different to IF and the role of IF in defence depends on the previous metabolic status of the plant and on the bacterial MAMP.

1. Introduction

Plants live in complex environments subject to continuous changes. Since they are sessile organisms, they survive to these changes with a plethora of chemical molecules known as secondary metabolites. Secondary metabolism provides plasticity to these changes (Van den Ende and El-Esawe, 2013), so identification of the factors responsible for these changes is key to control secondary metabolism in our profit.

To protect themselves against biotic agents, plants have a natural immune system that may be triggered by different agents (Dodds and Rathjen, 2010). Several classes of plant immunity have been described, namely systemic acquired resistance (SAR) and induced systemic resistance (ISR). Both confer resistance to a broad spectrum of pathogens in plant parts that were not infected and distant to initial infection site (Van Loon et al., 1998). However, while SAR is activated after infection by a necrotizing pathogen and is associated with local and systemic increase of SA, ISR is triggered by specific microbes, or MAMPs, and is mediated by JA and ET

Abbreviations: ACC, 1-amino-cyclopropane-1-carboxylic Acid; DF, daidzein family; ET, ethylene; GF, genistein family; IF, isoflavones; ISR, induced systemic resistance; MAMPs, microbe associated molecular patterns; PGPR, plant growth promoting rhizobacteria.

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(Ramos Solano et al., 2008; Pieterse et al., 2012); MAMPs are specific bacterial determinants (microbe associated molecular patterns (MAMPs) (Erbs and Newman, 2012). When MAMPs trigger plant metabolism, plant growth regulators' balance is affected and plant immune responses are activated (Denance et al., 2013).

The enormous variability of these MAMPs relays on the evolutionary relationship between the plant and its potential pathogens.

However, disease resistance has a fitness cost and sometimes growth is compromised, therefore, there is a fine-tune of defensive metabolism (Van Hulten et al., 2006; Ruhmann et al., 2013). Before pathogen challenge, elicited plants are in a unique physiological state called "priming" that is induced in the plant (Conrath, 2011). In the "primed" state plants are able to develop a more rapid activation, stronger, or both at the same time, defensive responses after exposure to stresses, among which is the presence of a pathogen (Conrath et al., 2006). This enhanced response involves accumulation of molecules (phytoanticipins) that are immediately transformed in defensive compounds (phytoalexins) upon challenge (Boue et al., 2009). Consequently, the use of PGPR or their MAMPs to trigger secondary metabolism has a great potential, since the metabolic pathways involved in the synthesis of defence compounds (phytoalexins), are also relevant in the pharmaceutical or food industry; therefore, systemic induction is a biotechnological tool of great added value (Boue et al., 2009; Capanoglu, 2010; Ramos-Solano et al., 2010).

Soybean contains significant amounts of isoflavones, daidzein and genistein, either as the aglycone or as its glucosyl or malonylglucose conjugates that are beneficial for human health. Benefits depend on the total IF content and in the qualitative IF profile (Isanga and Zhang, 2008). Isoflavones are synthesized by a branch of the phenylpropanoid pathway involved in defence (Fig. 1) (Dixon et al., 2013), its synthesis is inducible and their antimicrobial activity has been demonstrated (Yu and McGonigle, 2005).

In view of the above, it seems interesting to find effective MAMPs able to increase IF contents in soybean with a double aim: to enhance plant protection and to obtain a food product with increased IF content suitable to improve human health. In a previous study, four PGPR strains were selected for the different way to alter IF content in soybean seedlings (Ramos-Solano et al., 2010). The aim of this study was to evaluate the ability of these strains to induce systemic defence mechanisms in soybean plants and to determine if plant protection involved IF. To achieve the objectives, the ability of these strains to protect against the soybean pathogen *X. axonopodis* pv. *glycines* was assessed studying the pathway involved in disease resistance through a biochemical marker (ET), and evaluating the effects on growth and photosynthesis as physiological markers. Finally, IF levels were studied to establish the relation between IF profile and protection caused by the bacteria and the role of IF as metabolic markers of induction.

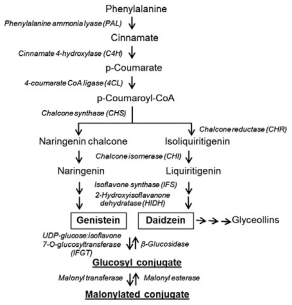

Fig. 1. Simplified diagram of isoflavone biosynthesis in soybean showing key intermediates and enzymes.

2. Materials and methods

2.1. Plant material

Glycine max var. Osumi plants belong to the so-called short cycle and were kindly provided by Dra. Rodríguez Navarro at (CIFA) Las Torres-Tomejil, Sevilla.

2.2. Bacterial strains

The PGPR strains used in this study were *Pseudomonas fluorescens* N21.4, *Stenotrophomonas maltophilia* N5.18, *Chryseobacterium balustinum* Aur9 and *Curtobacterium* sp. M84. These strains were selected in previous studies based on their ability to stimulate differentially isoflavone metabolism in soybean seedlings (Ramos-Solano et al., 2010) and their ability to stimulate growth and defensive metabolism in different plant species (Ramos-Solano et al., 2010).

The leaf pathogen *Xanthomonas axonopodis* pv. *glycines* (LMG7488) was used for challenge inoculation. This strain was obtained from the BCCM™ (The Belgian Co-ordinated Collections of Micro-organisms).

All strains were maintained at −80 °C in nutrient broth (Pronadisa) amended with 20% glycerol.

2.3. PGPR and pathogen inoculations

Inoculum was prepared by streaking strains from −80 °C onto plate count agar (PCA) plates, incubating plates at 28 °C for 24 h, and scraping bacterial cells off plates into sterile 10 mM SO_4Mg buffer. PGPR inoculation was done by soil drench with a bacterial density such to achieve 10^8 cfu mL^{-1} substrate (250 mL pots). A control group was inoculated with 10 mM SO_4Mg buffer.

X. axonopodis pv. *glycines* (LMG7488) was applied by foliar spray at 10^9 cfu mL^{-1} in a moist chamber. Controls groups were mock-inoculated with 10 mM SO_4Mg buffer.

2.4. Cultivation of plants

G. max cv. Osumi seeds were sterilized in 70% ethanol by stirring for 1 min, 5% sodium hypochlorite for 6 min, and five washes with distilled water. Then, seeds were pre-germinated in 1% European Bacteriological Agar (Pronadisa) plates at 30 °C, in the dark and at 60% relative humidity in a growth chamber (Sanyo MLR-350H, Japan). Two hundred and fifty pre-germinated seeds (two-day-old) were transplanted to 250 mL pots filled with sterile vermiculite. Experiments were conducted in a greenhouse with the following conditions: natural photoperiod (16/8 h light/dark), $28 \pm 2/20 \pm 2$ °C, at 70% relative humidity. Irrigation was applied by spraying with water for 3 min, 3 times a day (272.5 mL m^{-2} min^{-1}) except 1 day before and after PGPR inoculation and pathogen infection. In addition, there were three irrigations (at 6, 14 and 22 days after transplant) with 20 mL of 50% nutrient solution (Rigaud and Puppo, 1975) supplemented with 1 mL L^{-1} of a microelement solution (Gibson, 1963); N supply was ensured with 200 mg of N (KNO_3) per plant throughout the experiment.

2.5. Experimental set up

Two applications with PGPR were made, at transplant and 20 days after. Plants ($n = 250$) were divided into 5 groups ($n = 50$) and then inoculated with the four bacterial strains (N21.4, Aur9, N5.18, M84) leaving a group of plants as non-inoculated controls. Sixteen hours after the second inoculation, six plants from each group were harvested to determine ethylene production as a metabolic marker