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Transcription analyses of *GmICHG*, a gene coding for a β-glucosidase that catalyzes the specific hydrolysis of isoflavone conjugates in *Glycine max* (L.) Merr

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ABSTRACT

Isoflavone conjugate-hydrolyzing β-glucosidase (GmICHG) of soybeans [*Glycine max* (L.) Merr.] catalyzes the specific hydrolysis of isoflavone conjugates (β-7-*O*-(malonyl)glucosides of isoflavones) to produce free isoflavones. In this study, changes in the transcription levels of *GmICHG* in the individual organs of soybean seedlings (cv. Enrei) in response to microbial infection and abiotic stresses were analyzed and compared with those of genes coding for 2-hydroxyisoflavanone synthase (*GmIFS*) and isoflavone 7-*O*-glucosyltransferase (*GmIF7GT*). *GmICHG* was originally expressed in abundance only in the roots and at low levels only in the other organs. The transcription of *GmICHG* in the roots and other organs was suppressed upon infection of the roots by *Phytophthora sojae*. Upon wounding of the cotyledon, a transient long-distance up-regulation of *GmICHG* transcription in the roots was observed; upon fungal infection in the cotyledon, however, a delayed elevation of *GmICHG* transcription took place in the roots with the maximum at 10 h after the infection. Such long-distance up-regulation patterns were not observed with either *GmIFS* or *GmIF7GT*. The transcription levels of *GmICHG* remained essentially unchanged upon treatment of the roots with *Bradyrhizobium japonicum*. The transcription of *GmICHG* in the roots was also sensitive to a variety of stresses on the roots, such as flooding, elicitation with yeast extract, drought, and treatment with plant hormones such as abscisic, salicylic, and jasmonic acids and ethylene.

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1. Introduction

Isoflavones (*e.g.*, **1a**, daidzein; **2a**, genistein; see Fig. 1) predominantly occur in Papilionoideae plants, where these flavonoids play very important roles in plant-microbe interactions. In the soybean (*Glycine max* (L.) Merr.), isoflavones are involved in defensive mechanisms against pathogen infection—**1a** serves as the precursor of the glyceollin phytoalexins [1,2] and **2a** displays antibiotic activity against pathogens such as *Phytophthora sojae*, which is an oomycete causing soybean root and stem rot [3,4]. **2a** is also involved in defense potentiation and the establishment of elicitation competence for the glyceollin response [1,5]. Moreover, **1a** and **2a** are exuded from the roots and serve as chemo-attractants for symbiotic microorganisms (*e.g.*, *Bradyrhizobium japonicum*) and inducers for bacterial production of nod factors [6]. Endogenous isoflavones also serve as *nod* gene inducers that are essential for nodulation in soybean roots [7].

In the soybean, **1a** and **2a** are derived from the corresponding (2*S*)-flavanones, *i.e.*, liquiritigenin and naringenin, respectively, which undergo 2-hydroxylation catalyzed by 2-hydroxyisoflavanone synthase (GmIFS), a microsomal cytochrome P450 enzyme [8–10]. The resultant products, 2,7,4′-trihydroxyisoflavanone and 2,5,7,4′-tetrahydroxyisoflavanone, then undergo dehydration to produce **1a** and **2a**, respectively, by the action of 2-hydroxyisoflavanone dehydratase (GmHID) [11] (Fig. 1). At least in part, **1a** and **2a** thus produced can be extracellularly secreted by the action of an ATP-binding cassette-type transporter and may be involved in the plant-microbe interactions mentioned above [12]. Isoflavonoids also accumulate in vacuoles in large amounts as their conjugated forms, 7-*O*-glucosides (**1b** and **2b**) and 7-*O*-6″-*O*-malonylglucosides (**1c** and **2c**), which are more water-soluble than the aglycons [6,13–15,33]. The first step of the isoflavone conjugation is 7-*O*-glucosylation, which is specifically catalyzed by the UDP-glucose:isoflavone 7-*O*-glucosyltransferase (GmIF7GT) [16] (Fig. 1).

Strong isoflavone-conjugate-specific β-glucosidase activity was identified in apoplastic fractions of the roots of soybean seedlings [17]. The results of previous studies have suggested the possible involvement of β-glucosidase activity in defensive mechanisms against pathogen infection in soybean cotyledons. For example, in cotyledons, the conjugates of daidzein (**1b** and **1c**) occur at a level much higher than the effective level of glyceollin, and are rapidly hydrolyzed during incompatible infection by *P. sojae* at the infection site [13]. Moreover, distal defense potentiation against *P. sojae*

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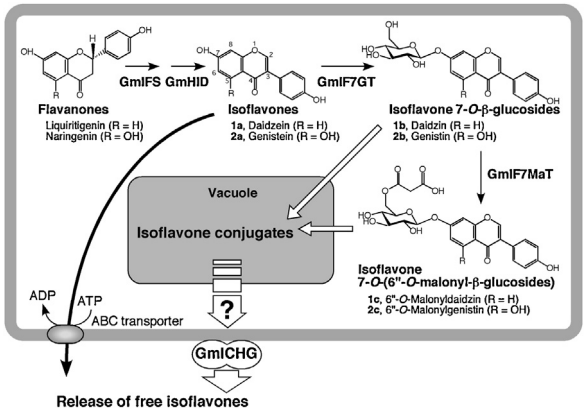

Fig. 1. Proposed pathways of isoflavone conjugate biosynthesis in soybean cells and extracellular release of free isoflavones. Abbreviations used are as follows: GmIFS, 2-hydroxyisoflavanone synthase; GmHID, 2-hydroxyisoflavanone dehydratase; GmIF7GT, UDP-glucose:isoflavone 7-*O*-glucosyltransferase; GmIF7MaT, malonyl-CoA:isoflavone 7-*O*-glucoside 6″-*O*-malonyltransferase; and, GmICHG, isoflavone conjugate-hydrolyzing β-glucosidase.

induced by wounding and elicitor treatment prior to fungal infection strongly correlates with levels of the conjugates (**2b** and **2c**) in cotyledons [18]. Thus, these isoflavone conjugates can be thought of as stored precursors of antimicrobial agents [13]—the formation and hydrolysis of the conjugates are proposed to occur under intricate regulation by the host and the pathogen. Moreover, the isoflavone conjugates appear to serve as the stored precursors of isoflavones involved in rhizobia-mediated nodulation [13]. In previous studies, when the root exudate was heated at 100 °C for 5 min immediately after collection, it was **1c** rather than **1a** [15] that was the major isoflavone identified in the exudate, which suggested the importance of specific β-glucosidase activity during the exudation of **1a** and **2a** from soybean roots.

We previously reported the molecular cloning and biochemical characterization of a β-glucosidase, *G. max* isoflavone conjugate-hydrolyzing β-glucosidase (GmICHG) (Fig. 1) [19]. This enzyme is a member of the glycoside hydrolase family 1 (GH1) and specifically acts on both 7-*O*-glucosides and 7-*O*-6″-*O*-malonylglucosides of isoflavones to produce **1a** and **2a**. Immunolocalization studies, as well as GFP-imaging analyses, showed that GmICHG is exclusively localized in the apoplastic space of seedling roots and in the cell wall of root hairs. On the basis of these observations, the involvement of GmICHG has been implicated in isoflavone-mediated biological processes, including defense responses against pathogens and rhizobia-mediated nodulation [13,14,17,19]. To further unravel the physiological functions of this enzyme, changes in *GmICHG* transcription levels in the individual organs of soybean seedlings in response to microbial infection (by *P. sojae* or by *B. japonicum*) and abiotic stresses were analyzed and compared with those of functionally related enzymes and proteins of soybean (GmIFS1, GmIF7GT, and/or a pathogenesis-related protein (GmPR1a, [20]).

2. Material and methods

2.1. Plant materials, microbial strains, and chemicals

The soybean seeds (cv. Enrei) used in this study were kindly provided by Prof. Kiwamu Minamisawa, Graduate School of Life Sciences, Tohoku University, Sendai, Japan. *B. japonicum* USDA110 is a stock culture of the Laboratory of Environmental Plant Microbiology, Graduate School of Life Sciences, Tohoku University. *P. sojae* (the Ps060619-3-1 isolate) was kindly provided by Dr. Joji Moriwaki, Hokuriku Research Center, National Agricultural Research Center, Niigata, Japan. The soybean plant (cv. Enrei) that was used in this study was susceptible to the Ps060619-3-1 strain. Isoflavonoids (**1a**, **1b**, **1c**, **2a**, **2b**, and **2c**) were purchased from Fujicco (Kobe, Japan). Acetonitrile and trifluoroacetic acid, both of HPLC-grade, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Yeast extract and salicylic acid (SA) were obtained from Nacalai Tesque (Kyoto, Japan). Abscisic acid (ABA) and ethephon were obtained from Sigma–Aldrich. Jasmonic acid (JA) and JA methyl ester (MeJA) were obtained from the Tokyo Chemical Industry (Tokyo, Japan).

2.2. Soybean cultivation

Uniform, unblemished, disease-free soybean seeds (cv. Enrei) were surface-sterilized with 70% ethanol for 30 s followed by 0.04% hypochlorite solution for 8 min. The seeds were sown in sterile vermiculite with a nitrogen-free plant nutrient solution [21] in sterile Leonard jar assemblies composed of two 300-ml plant boxes [22], and grown for 3 days at 25 °C in the dark. The plants were further cultivated in uncovered Leonard jar assemblies for 5 days at 25 °C under long-day conditions (16 h light and 8 h dark) in a plant growth cabinet (Plant Environmental Control System model KCLP-1400 II CT, Nippon Medical & Chemical Instruments, Osaka, Japan), which provided ~200 μmol photons m^{-2} s^{-1} of white light irradiation.

2.3. Infection by P. sojae

P. sojae (the Ps060619-3-1 isolate) was grown on a 20% clarified V8-juice agar (V8A) plate at 23 °C for 7 days in the dark. The soybean seeds were germinated and cultivated for 7 days with the nitrogen-free nutrient solution in sterile Leonard jar assemblies, as described in Section 2.2.

2.3.1. Infection of roots

The soybean seedling was carefully removed from the upper vessel of the jar, and the root system of the seedling was put on a *P. sojae*-grown V8A plate (Supplementary Fig. S1). Forceps were used to place the roots in contact with mycelia to facilitate fungal infection. After 0, 2, 5, 10, and 24 h, roots, hypocotyls, and a cotyledon of the seedlings (typically 5 samples) were collected and immediately frozen in liquid nitrogen (infected roots used for transcription analyses). The mock-inoculated control samples were likewise obtained, except that the root systems of the seedlings were placed on a sterile V8A plate. In the *P. sojae*-infected systems, pathological symptoms could be confirmed by observation of the water-soaked necrotic lesions throughout seedlings until 6 days after inoculation (Supplementary Fig. S1).

2.3.2. Infection of cotyledons

The cuticle layer (approximately 7 mm square in width) on the adaxial surface of a cotyledon of the seedlings was carefully removed, using a scalpel, to facilitate fungal infection. The seedlings were immediately inoculated by placing a V8A block (5 mm cube) with seven-day-old mycelia directly on the cuticle-removed portion of the cotyledon (Supplementary Fig. S1). The inoculated plants were incubated under long-day conditions in a plant growth cabinet as described above. After 0, 2, 5, 10, and 24 h, taproots, lateral roots, hypocotyls, and a cotyledon of the seedlings (typically 5 samples) were collected and immediately frozen in liquid nitrogen. The cotyledons without fungal inoculation were collected and used for transcription and flavonoid analyses. In the *P. sojae*-infected systems, pathological symptoms could be confirmed by observation of the water-soaked necrotic lesions at the cotyledon inoculated until 2 days after inoculation (Supplementary Fig. S1). The mock-inoculated control samples were likewise obtained, except that the V8A block without *P. sojae* mycelia was placed on the cuticle-removed cotyledon. It must be noted that the removal of the

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