Short title: β-Glucosidase increases isoflavones in rhizosphere

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Apoplast-localized β-glucosidase facilitates the root-to-soil delivery of isoflavones in soybean

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One sentence summary: In soybean, isoflavone glycosides stored in the roots are deglycosylated to aglycones by an apoplastic β-glucosidase, and this process supplies isoflavone aglycone to the rhizosphere.

Footnotes

Author contributions

H.M. and A.S. conceived and designed the research; K.Y. and A.S. supervised the experiments; A.K. screened and backcrossed *ichg* mutants; H.M., M.N., H.T., and A.S.

grew and sampled soybeans for the experiments; H.M., Y.Y., E.M., and A.S. performed the protein activity assays; H.M. and A.J.N. conducted RNA-seq experiments; H.M, S.Y., and Y.A. analyzed the transcriptomic data; H.M. performed the analysis of isoflavone contents, bacterial communities, and rhizobial gene expressions; H.M. and S.O. analyzed infection threads using fluorescent-tagged rhizobia; H.M., M.N., A.K., K.Y., and A.S. wrote the article with contributions from all the authors; A.S. agrees to serve as the author responsible for contact and ensures communication.

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Abstract

Plant specialized metabolites (PSMs) are often stored as glycosides within cells and released from the roots with some chemical modifications. While isoflavones are known to function as a symbiotic signal with rhizobia and to modulate the soybean rhizosphere microbiome, the underlying mechanisms of root-to-soil delivery are poorly understood. In addition to transporter-mediated secretion, the hydrolysis of isoflavone glycosides in the apoplast by an isoflavone conjugate-hydrolyzing β-glucosidase (ICHG) has been proposed but not yet verified. To clarify the role of ICHG in isoflavone supply to the rhizosphere, we have isolated two independent mutants defective in ICHG activity from a soybean high-density mutant library. In the *ichg* mutants, the isoflavone contents and composition in the root apoplast and root exudate significantly changed. When grown in a field, the lack of ICHG activity significantly reduced isoflavone aglycone contents in roots and the rhizosphere soil, although the transcriptomes showed no distinct differences between the *ichg* mutants and WTs. Despite the change of isoflavone contents and composition in the root and rhizosphere of the mutants, root and rhizosphere bacterial communities were not distinctive from those of the WTs. Root bacterial communities and nodulation capacities of the *ichg* mutants did not differ from the WTs under nitrogen-deficient conditions, either. Taken together, these results indicate that ICHG facilitates the delivery of isoflavones from soybean roots to the rhizosphere and that deglycosylation in the root apoplast is a crucial reaction by which to enhance PSM accumulation in the rhizosphere.

Introduction

It is predicted that plant species produce over one million different organic compounds (Afendi et al., 2012). It has also been found that approximately 30 %–60 % of net fixed carbon is transferred to plant roots and approximately 40 %–90 % of this transferred carbon is released into the rhizosphere, depending on the plant species, age, and environmental conditions (Lynch and Whipps, 1990). Root exudates contain amino acids, sugars, organic acids, fatty acids, vitamins, nucleotides, and specialized (secondary) metabolites (Vives-Peris et al., 2020), and these plant-borne metabolites affect rhizosphere soil properties, such as aggregation, pH, and the microbiome (Ehrenfeld et al., 2005). Plant specialized metabolites (PSMs) have gained attention as they are critical for improving plant nutrition, repelling pathogens and pests, and modulating abiotic stress tolerance (Massalha et al., 2017; Pascale et al., 2019; Abedini et al., 2021; Jacoby et al., 2021; Pang et al., 2021). Recent studies have demonstrated that PSMs significantly contribute to modulations in the composition and function of the root and rhizosphere microbiome, influencing both plant growth and fitness. Flavonoids, one of the common PSMs in the rhizosphere, function as a signaling molecule to induce rhizobial Nod factors and initiate legume-rhizobia symbiosis (Subramanian et al., 2007), and as iron chelators to promote iron availability (Cesco et al., 2010). Flavonoids can also enrich specific bacterial families in the rhizosphere microbiota, such as *Comamonadaceae* in soybean and *Oxalobacteraceae* in maize, improving maize performance in nitrogen-deficient conditions (Okutani et al., 2020; Yu et al., 2021).

Once flavonoids are synthesized at the surface of the endoplasmic reticulum in the cytosol, they are transported into the vacuole for storage (Marrs et al., 1995; Zhao and Dixon, 2010). Due to the toxicity, instability, and/or insolubility of aglycones in the cells, flavonoids exist as glycosides in the vacuole (Matern et al., 1983; Le Roy et al., 2016). β-Glucosidases, often found in the plastid and apoplast, hydrolyze a variety of glycosides into aglycones when glycosides are delivered from the vacuole for defense or the development of symbiotic relationships (Morant et al., 2008; Ketudat Cairns and Esen, 2010). In soybeans, isoflavone aglycones such as daidzein and genistein serve as initiation signals for symbiosis with *Bradyrhizobium diazoefficiens* and *Ensifer fredii* (Kosslak et al., 1987; Pueppke et al., 1998), and also affect the assembly of the rhizosphere microbiome (Okutani et al., 2020).

Both plasma membrane-localized ATP-binding cassette (ABC) transporters and multidrug and toxic compound extrusion (MATE) transporters mediate the secretion of (iso)flavonoid aglycones to the rhizosphere (Sugiyama et al., 2007; Biala et al., 2017; Biała-Leonhard et al., 2021). In addition, isoflavone glycosides stored in the vacuole are proposed to be secreted to the apoplast, since isoflavone conjugate-hydrolyzing β-glucosidase (ICHG) hydrolyzes these glycosides to aglycones in the apoplast (Fig. 1) (Suzuki et al., 2006).

ICHG is a homodimeric glycoprotein, expressed in the roots, especially from the stele to lateral root hairs, and localized in the cell walls and intercellular space (Suzuki et al., 2006; Yoo et al., 2013). ICHG efficiently hydrolyzes isoflavone malonylglucosides

and glucosides (Suzuki et al., 2006). Previous studies have suggested that there is a relationship between *ICHG* gene expression and isoflavone secretion during the different developmental stages of soybean and its diurnal regulation when hydroponically grown. From the vegetative to reproductive stages, *ICHG* gene expression levels were found to decrease while the isoflavone glycoside contents in the hydroponic culture increased (Sugiyama et al., 2016). The expression levels of the isoflavone biosynthetic genes and candidate isoflavone transporter genes increased around noon each day and then decreased around midnight, but the isoflavone aglycone content in the hydroponic medium remained constant (Matsuda et al., 2020). The gene expression of *ICHG* exhibited the opposite diurnal pattern against the isoflavone biosynthetic genes and candidate transporters, suggesting that ICHG degrades the glycosides secreted from the vacuole to complement the decreased transporter-mediated secretion of isoflavone aglycones during the night (Matsuda et al., 2020). Despite these gene expression analyses and enzymatic characterizations, the physiological function of ICHG remains unknown. In this study, we employed soybean mutants defective in ICHG to clarify how ICHG contributes to the accumulation of isoflavones in the rhizosphere and their interactions with soil bacteria.

Results

Development of *ichg* mutants

We screened *ichg* mutants from a high-density mutant library, generated using

147 an ethyl methanesulfonate (EMS) treatment twice, via an amplicon sequencing method
148 (Tsuda et al., 2015). Two *ichg* mutants, with missense (Glu420Lys hereafter *ichg-1*) and
149 nonsense (Gln232stop hereafter *ichg-2*) mutations, respectively, in the *ICHG* gene
150 (*Glyma.12G053800*), were identified from the 1,536 mutants in the library (Fig. 2A).
151 These mutants were backcrossed with the wild-type cultivar Enrei, F_1 plants were
152 subjected to self-pollination, and seeds obtained from the F_2 plants (the missense mutant-
153 derived homozygous *ichg-1* and homozygous wild-type (WT)-1 plants; the nonsense
154 mutant-derived homozygous *ichg-2* and homozygous WT-2 plants) were used in the
155 following experiment (Fig. 2B).

156 Heterologously expressed ICHG in *Escherichia coli* specifically degrades
157 isoflavone glycosides, and malonylglucoside has been identified as the best substrate
158 (Suzuki et al., 2006). We evaluated malonyldaidzin degradation activity using apoplastic
159 crude proteins extracted from the roots of the F_2 progenies. The ICHG activity in the
160 crude protein from the soybeans with either the *ichg-1* or *ichg-2* alleles was below the
161 detection limit (Fig. 2C), while that of the WTs was apparent. These results confirmed
162 that the activity of ICHG in the apoplastic crude proteins extracted from both *ichg* mutants
163 was completely lost, and thus, these mutants could be utilized in further analysis.

164

165 **Isoflavone contents in the root apoplastic fraction and hydroponic medium of *ichg***
166 **mutants**

167 To verify the effects of the ICHG defects in the root apoplast where ICHG is

localized, we analyzed isoflavone aglycones and glycosides in the root apoplastic fraction collected from 1-week-old soybean seedlings. Daidzein levels were much lower in the apoplastic fraction of *ichg-1* and *ichg-2* than the WT plants (p = 0.008) (Fig. 3A). Daidzein levels in the whole roots of *ichg-1* and *ichg-2* were also lower than those of the WTs (*ichg-1*, p = 0.009; *ichg-2*, p = 0.035) (Fig. 3C). Genistein levels in the apoplastic fraction and roots of the *ichg* mutants showed a declining trend when compared with the WT plants (apoplastic fluids: *ichg-1*, p = 0.178; *ichg-2*, p = 0.103; whole roots: *ichg-1*, p = 0.006; *ichg-2*, p = 0.020) (Fig. 3A, C). Contents of the daidzin, genistin, malonyldaidzin, and malonylgenistin were significantly higher in the root apoplastic fractions of the *ichg* mutants than those of the WTs, while their contents in the roots were comparable between the *ichg* mutants and WTs (Fig. 3A, C). In addition, total isoflavone contents in the apoplastic fractions were also considerably higher in the *ichg* mutants than the WTs (*ichg-1*, p = 0.062; *ichg-2*, p = 0.049) (Fig. 3B); while no clear difference was observed in the total isoflavone content of the whole roots (Fig. 3D).

As ICHG defects affected isoflavone contents in the root apoplast, we investigated the changes of isoflavones in the root exudate of *ichg* mutants in the same growth stage. In the hydroponic medium of *ichg-1* and *ichg-2*, the contents of isoflavone glycosides increased, while aglycone contents did not show a significant change from the WTs (Fig. 4A). The total isoflavone contents in the medium of *ichg* mutants were higher than in WTs due to the increase of isoflavone glycosides (*ichg-1*, p = 0.133; *ichg-2*, $p <$ 0.001) (Fig. 4B). Total isoflavone contents in roots were comparable between mutants

and WTs, while isoflavone aglycone contents showed decreasing trends in the mutants (daidzein: *ichg-1*, $p = 0.208$; *ichg-2*, $p = 0.013$; genistein: *ichg-1*, $p = 0.161$; *ichg-2*, $p = 0.006$) (Fig. 4C, 4D). Together, these results indicate that ICHG involves in the isoflavone release from soybean roots.

Transcriptome analysis on field-grown *ichg* mutants

To assess the impact of the *ichg* mutations on the soybean transcriptomes, we conducted RNA-seq analysis using total RNA collected from 7-week-old field-grown soybean leaves and roots. The *ICHG* gene expression levels in roots were approximately more than 10-fold higher than those in leaves and they showed no significant differences between the *ichg-1* and WT-1 in both organs (Fig. 5A); whereas, in *ichg-2* the expression level of *ICHG* was significantly lower than in WT-2 (leaf, $p = 0.002$; root, $p = 0.005$) (Fig. 5A). Principal component analysis (PCA) of the transcriptomic data showed no statistically distinctive characteristics in any of the genotypes (Fig. 5B, Supplemental Table S1). The numbers of differentially expressed genes (DEGs) between WT-1 and *ichg-1*, and WT-2 and *ichg-2* are provided in Supplemental Table S2. No common genes were found in the DEGs when comparing WT-1 to *ichg-1* and WT-2 to *ichg-2* (Supplemental Table S3, S4). As for the isoflavone biosynthetic genes, only the expression of the *isoflavone reductase homolog 2* (*Glyma.04G012300*) was found to be up-regulated in *ichg-1* leaves (Supplemental Table S3) while its gene expression did not increase in *ichg-2*. Taken together, these results demonstrate that the expression level of

ICHG is more pronounced in the roots than in the leaves and the loss of ICHG activity did not largely affect the soybean transcriptome or isoflavone metabolism.

Isoflavone contents in field-grown *ichg* mutants

To examine how ICHG defects affect the isoflavone contents and composition in field grown soybeans, we extracted isoflavone aglycones and glycosides from the leaves, roots, and rhizosphere soil. In the leaves, daidzein and genistein were not detected (Fig. 6A), and their glucosides and malonylglucosides contents showed no significant differences between the WT-1 and *ichg-1* or WT-2 and *ichg-2* (Fig. 6A, B). In roots, the daidzein and genistein contents in *ichg-1* and *ichg-2* were remarkably lower than those in WT-1 and WT-2, respectively (daidzein: *ichg-1*, $p = 0.025$; *ichg-2*, $p = 0.024$; genistein: *ichg-1*, $p = 0.009$; *ichg-2*, $p = 0.005$) (Fig. 6C). The isoflavone glycosides contents, including daidzin, malonyldaidzin, genistin, and malonylgenistin did not significantly differ between the *ichg* mutants and WTs (Fig. 6C). The total isoflavone content in the roots of both *ichg* mutants was slightly higher than that of their corresponding WTs (Fig. 6D), but the results were not statistically significant.

In the rhizosphere soil, the amount of daidzein and genistein in *ichg-2* was significantly lower than in WT-2 (daidzein, $p = 0.003$; genistein, $p = 0.001$) (Fig. 6E). The isoflavone aglycone content in *ichg-1* was also decreased when compared with WT-1 (Fig. 6E). As the contents of the isoflavone glycosides were not significantly increased in the mutants, the total amount of isoflavones in the rhizosphere soil of *ichg-1* and *ichg-*

231 *2* was reduced to approximately half of that in the rhizosphere soil of the WT plants (Fig.
232 6E, F). There were only trace amounts of the isoflavones for both aglycones and
233 glycosides detected in the bulk soil (Fig. 6E, F). These results suggest that ICHG
234 facilitates the root-to-soil delivery of isoflavones.
235
236 **Endosphere and rhizosphere bacterial communities of field-grown *ichg* mutants**
237 Isoflavones enrich *Comamonadaceae* in the soil microbiome and alter the
238 bacterial communities to resemble those of the soybean rhizosphere (Okutani et al., 2020).
239 To evaluate the effects of the decrease of isoflavone aglycone in the apoplast and
240 rhizosphere on the microbiome, we analyzed both the endosphere and rhizosphere
241 bacterial communities using amplicon sequencing of the V4 region of the 16S rRNA.
242 Rarefaction curves showed a similar number of the observed sequence variants in *ichg*
243 mutants and their corresponding WTs (Supplemental Fig. S1A). The principal coordinate
244 analysis (PCoA) of the weighted and unweighted UniFrac distance toward all samples
245 exhibited clear distinctions between the endosphere, rhizosphere, and bulk soil bacterial
246 communities (Permutational multivariate analysis of variance (PERMANOVA), q =
247 0.001) (Supplemental Fig. S1B, Table S5). As for the endosphere bacterial communities,
248 the PCoA of the weighted and unweighted UniFrac distance showed no clear distinctions
249 among the genotypes examined (Fig. 7A, Supplemental Table S6). Similarly, no
250 significant differences were observed among the rhizosphere bacterial communities of
251 the examined genotypes (Fig. 7B, Supplemental Table S7). The relative abundance of

Comamonadaceae in the *ichg-2* endosphere microbiome was lower than in WT-2 (p = 0.013) but there was no significant difference between WT-1 and *ichg-1* (Supplemental Fig. S1C). In the rhizosphere, the relative abundance of *Comamonadaceae* did not differ among the genotypes (Supplemental Fig. S1C). The numbers of enriched and depleted bacterial families between WT-1 and *ichg-1*, and WT-2 and *ichg-2* are provided in Supplemental Table S8. No common family was found when comparing *ichg-1* to WT-1 and *ichg-2* to WT-2 except for *Solimonadaceae*, which was depleted in the rhizosphere of the *ichg* mutants, however, the relative abundance of *Solimonadaceae* was <0.1 % in all samples (Supplemental Table S9, S10). These results suggest that ICHG is not crucial for the assembly of the soybean root and rhizosphere bacterial communities.

Root transcriptome, isoflavone contents, and bacterial communities of *ichg* mutants under nitrogen-deficient condition

Under a nitrogen-limited environment, isoflavone biosynthetic genes are up-regulated in legume roots and more isoflavone aglycones are secreted from roots (Coronado et al., 1995; Sugiyama et al., 2016). To investigate the roles of ICHG when isoflavone production and secretion are highly active, we grew *ichg* mutants both under nitrogen-sufficient (N+) and nitrogen-deficient (N-) conditions, sampled their roots at V3 stage when ICHG expression is high (Sugiyama et al., 2016). The results of PCA on the RNA-seq data showed two-separated clusters of N+ and N- samples (PERMANOVA, q = 0.005) (Fig. 8A, Supplemental Table S11). The DEGs comparing N+ and N- conditions

are listed in Supplemental Table S12. Under N- condition, genes involved in isoflavone biosynthesis were up-regulated as observed in the previous studies (Chu et al., 2017; Sun et al., 2021; Nezamivand-Chegini et al., 2022) (Supplemental Figure S2, Table S12). The expression levels of *ICHG* in N- conditons did not increase but showed a declining trend in Enrei WT (p = 0.101) and *ichg-1* (p = 0.047) (Fig. 8B). In *ichg-2*, *ICHG* expression levels were significantly lower than WT-2 (N+, p = 0.004; N-, p = 0.023) (Fig. 8B), which were the same results with field-grown soybean leaves and roots (Fig. 5A). The contents of daidzein and genistein in N- roots showed upward trends from N+ roots in both *ichg* mutants and WTs (Fig. 8C). Comparing *ichg* mutants with WTs, the difference in the isoflavone aglycone contents was more pronounced in N- than N+ roots (Fig. 8C). As for isoflavone glycosides, no significant differences were observed except for the considerable increase in the malonyldaidzin contents in Enrei WT under N- conditions (Tukey's HSD test, p = 0.018) (Fig. 8C). These results suggest that ICHG is not involved in the increase of the isoflavone contents in soybean roots under N- condition, however, the effect of the *ichg* mutation on the isoflavone aglycone contents becomes more apparent under N- condition than N+ condition.

In endosphere bacterial communities, the number of observed sequence variants did not differ among genotypes and/or nitrogen conditions (Supplemental Fig S3A). Unweighted and weighted UniFrac-based PCoA results showed a clear distinction between root bacterial communities under N+ and N- conditions (PERMANOVA, q = 0.001) (Supplemental Fig. S3B, Table S13) and 26 genera were enriched and 13 genera

were depleted under N- conditions, regardless of the ICHG defects (Supplemental Table S16, S17). Relative abundance of an unannotated genus of *Comamonadaceae* and *Bradyrhizobium* showed upward trends under N- conditions (Fig. 8D, Supplemental Table S16, S17). No common genus was found when comparing *ichg* mutants and WTs under both N+ and N- conditions (Supplemental Table S18, S19). These results suggest that ICHG is not pivotal for the assembly of the soybean root bacterial communities under both N+ and N- conditions.

Effect of the *ichg* mutation on nodule formation

We analyzed whether the decrease in isoflavone aglycones in the apoplast and rhizosphere affects rhizobial nodulation. Both *ichg* mutants and WTs were grown with *B. diazoefficiens* USDA 110 under N- conditions for 4 weeks. Shoot fresh weights did not show any statistically significant differences among WT-1, WT-2, *ichg-1,* and *ichg-2* (Fig. 9A). The root dry weight of *ichg-1* was higher than that of WT-1 ($p = 0.029$) (Fig. 9B). The nodule number of *ichg-2* was higher than that of WT-2 ($p = 0.043$), but there was no significant difference between WT-1 and *ichg-1* (Fig. 9C). Nodule weights were comparable across WT-1, WT-2, *ichg-1*, and *ichg-2* (Fig. 9D). The morphology of infection threads also appeared to be similar among all genotypes (Supplemental Fig. S4). Together, these results suggest that ICHG is not an essential component of the nodulation process.

Discussion

We obtained *ichg* missense and nonsense mutants by screening a soybean high-density mutant library. The *ichg-2* mutant harbored a nonsense mutation (Gln232stop) and the *ichg-1* mutant harbored a missense mutation (Glu420Lys), which resulted in the complete loss of ICHG activity, as this glutamic acid is one of two highly conserved glutamic acids among the glycosyl hydrolase family 1 β-glucosidases and a proposed catalytic residue (Barrett et al., 1995). Isoflavone aglycone contents were reduced in the root apoplastic fraction and rhizosphere soil of the *ichg* mutants; however, there was no remarkable change of transcriptomes in the *ichg* mutants both in the field and in pots of nitrogen deficiency. The expression of isoflavone biosynthetic genes and candidate transporter genes were comparable between *ichg* mutants and WTs and were up-regulated in nitrogen-deficient conditions in both *ichg* mutants and WTs (Supplementary Fig. S2, S5) (Matsuda et al., 2020). These results suggest there are little feedback or feedforward regulations derived from the effect of *ichg* mutation. Despite a reduction in the aglycone content of the roots and rhizosphere of the *ichg* mutants, there were no resulting common characteristics in the endosphere or rhizosphere bacterial communities or a specific nodulation phenotype. This was probably because the changes in the aglycone contents of the endosphere and rhizosphere were not drastic enough to affect the bacterial communities in the environment when other metabolites were present. For nodulation, isoflavone aglycone daidzein and genistein have been regarded as the major components of soybean root extracts responsible for inducing *nod* genes in *B. diazoefficiens* (Kosslak

et al., 1987). A recent study by Ahmad et al. (2021) suggested that isoflavone malonylglucosides also induce Nod factor production based on the analysis of over-expression and knockdown mutants of an isoflavone malonyltransferase, GmMaT2. Our analysis using isoflavone standard chemicals showed the slight induction of *nodD1* gene, but not *nodA* nor *nodY* genes, by malonyldaidzin (Supplemental Fig. S6), which is consistent with the previous study showing that isoflavone glycosides can induce *B. diazoefficiens* (*B. japonicum*) *nodD1* gene but not *nodYABCSUIJ* operon (Smit et al., 1992). These results suggest that the changes in isoflavone contents and composition in the *ichg* mutants do not affect the efficacy of nodule formation.

Isoflavone aglycones are known to be secreted from soybean roots and function in the rhizosphere but their underlying secretion mechanisms are poorly understood (Hassan and Mathesius, 2012; Sugiyama, 2021). While plasma membrane-localized ABC and MATE transporters mediate the export of isoflavone aglycones (Sugiyama et al., 2007; Biała-Leonhard et al., 2021), there is currently no evidence to explain how apoplast-localized β-glucosidase ICHG contributes to isoflavone supply to the rhizosphere. In general, glycosides are unstable in the soil, and those secreted from the roots are hydrolyzed to form aglycones within a few hours by soil bacterial enzymes, which leads to the assumption that ICHG has a minor role in the accumulation of isoflavones in the rhizosphere (Kong et al., 2007; Weir et al., 2010; Sugiyama et al., 2017). However, isoflavone content was significantly decreased in the rhizosphere of *ichg* mutants, demonstrating that the ICHG-mediated hydrolysis of isoflavone glycosides into

aglycones is pivotal for isoflavone supply to the rhizosphere (Fig. 10). Isoflavone glycosides in the apoplastic fraction and the hydroponic medium were significantly increased in *ichg* mutants. While isoflavone glycoside contents showed upward trends in the roots of field-grown *ichg* mutants, those in the rhizosphere soil of field-grown *ichg* mutants were not increased. One possible explanation for these results is the difference in the hydrophilicity between isoflavone aglycones and glycosides. In the apoplast of *ichg* mutants, isoflavones mainly exist as glycosides. The differences in hydrophobic and/or hydrophilic interactions in cell wall or mucilage may affect the delivery of isoflavones from root to rhizosphere soil.

PSMs often accumulate in vacuoles in their glycosylated forms and are secreted into the rhizosphere in their partially or fully de-glycosylated forms (Grubb and Abel, 2006; Frey et al., 2009; Neal et al., 2012; Strehmel et al., 2014; Stringlis et al., 2018; Tsuno et al., 2018). In *Arabidopsis*, coumarin glucoside scopolins are accumulated in roots and their aglycones, scopoletins, are secreted from roots under iron-limited conditions, in which these coumarins modulate the rhizosphere microbiota for systemic resistance and iron uptake (Stringlis et al., 2018). An *Arabidopsis* β-glucosidase, BGLU42, predicted to be localized in plastids, hydrolyzes scopolins to form scopoletins (Hooper et al., 2017). BGLU42 is required for the release of scopoletins into the rhizosphere, as *bglu42* mutants were found to secrete fewer scopoletins and instead accumulated larger amounts of scopolins in the roots (Zamioudis et al., 2014; Stringlis et al., 2018). Benzoxazinoids and glucosinolates are also found mostly in their

deglycosylated forms in the rhizosphere (Neal et al., 2012; Strehmel et al., 2014), but whether de-glycosylating enzymes are involved in their secretion, and which specific enzymes may be involved, requires further investigation. The present study sheds light on the previously unrecognized role of apoplast-localized β-glucosidases in relation to the accumulation of PSMs in the rhizosphere. Since there are multiple secretory routes that bring PSMs to the rhizosphere, it is important to elucidate how each route functions coordinately under various stress and developmental conditions. Further studies on β-glucosidases, other de-glycosylating enzymes, and other proteins related to PSM secretions will enable us to better understand the dynamics and functions of PSMs in the rhizosphere.

Materials and methods

Chemicals and plant materials

Malonyldaidzin and malonylgenistin were purchased from Nagara Science. All other chemicals were purchased from Wako Pure Chemical Industries Ltd. or Nacalai Tesque Inc., unless otherwise stated. Seeds of WT soybean cv Enrei were purchased from Tsurushin Syubyo, Matsumoto, Japan.

Screening *ichg* mutants from an EMS mutant library

The *ichg* mutants were obtained from a high-density mutant library of the cultivar Enrei according to a previously described amplicon sequencing method (Tsuda

et al., 2015). This library consisted of DNA and seeds from 1,536 EMS-induced mutant plants. Amplicons of the *ICHG* gene (*Glyma.12G053800* for Gmax_275 reference) which were approximately 6,700 bp, were amplified from 384 bulk DNA samples, each consisting of material from four mutant plants. PCR reaction mixtures (10 μL) contained 0.2 μL of template DNA from each of the 384 DNA pools, 2 μL of 5× PrimeSTAR GXL Buffer (Takara Bio, Kusatsu, Japan), 1.0 μL of PrimeSTAR GXL DNA Polymerase (1.25 U/μL), 0.8 μL of 2.5 mM dNTPs, and 0.1 μL of each of the 20 μM forward and reverse primers [ICHG-AS_F (5′-aatttggaatccgtgagtttcttgtga-3′) and ICHG-AS_R (5′-taataattcccgtcttgctttgtgctt-3′)]. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: initial denaturation for 5 s at 98 ℃; 30 cycles of denaturation for 10 s at 98 ℃, annealing for 15 s at 68 ℃, and extension for 7 min 35 s at 68 ℃; and a final extension for 30 s at 68 ℃. Four PCR samples were mixed to prepare 96 PCR amplicon pools. The dual index sequencing library was prepared using a Nextera™ XT DNA Sample Preparation Kit and Nextera™ XT Index Kit (Illumina, San Diego, CA, USA), both according to the manufacturers' instructions. Paired-end sequencing data was obtained on a MiSeq™ platform using a MiSeq™ v2 500-cycle kit (Illumina) with the default parameters. Read mapping and variant detection of the 96 PCR amplicon pools was conducted using CLC Genomics Workbench software (CLC Bio, Aarhus, Denmark) with the workflow and batch processing tools, according to the parameter settings described by Tsuda et al. (2015). PCR amplicon pools that contained missense (Glu420Lys hereafter *ichg-1*) or

nonsense (Gln232stop hereafter *ichg-2*) mutations in the *ICHG* gene were identified using the variant detection procedure (Tsuda et al., 2015). The plants in which the mutations occurred were identified by directly sequencing each of the 16 original DNA samples from the PCR amplicon pool. Direct sequencing was conducted using primer pairs (5′-attataatgcaggccgcttcagtttg-3′ and 5′-agagcttcttccacggaaagggttg-3′ for *ichg-1*; and 5′-ttgatatgattacaagttgttgagctttg-3′ and 5′-cttagtcttgtacacatgaacagcagc-3′ for *ichg-2*), a BigDye Terminator v3.1 Cycle Sequencing Kit, and an ABI 3730xl genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. The mutants *ichg-1* and *ichg-2* were backcrossed with the WT cultivar Enrei, and the self-pollinated F_2 progenies were used in the following experiment. The genotype of the F_2 progenies was determined by sequencing the amplicons obtained using a primer pair (5′-aggtctaagggtacatttgtag-3′ and 5′-tcttccacggaaagggttg-3′ for *ichg-1*; 5′-actaaaaatgttcgaaattcg-3′ and 5′-caactcaaagttgtgtgagaaaca-3′ for *ichg-2*). The seeds from the F_2 plants that were homozygous for the mutants or wild alleles (*ichg-1* and WT-1; *ichg-2* and WT-2) were used in the following experiments.

Extraction of apoplastic crude proteins

Soybean seeds (Enrei WT, WT-1, *ichg-1*, WT-2, and *ichg-2*) were sterilized with chlorine gas for 3 hours and germinated in plant boxes (AGC Techno Glass, Haibara, Japan) filled with autoclaved vermiculite and water (all sterilization and germination methods were the same hereafter unless otherwise specified). The 1-week-old seedlings

were rinsed and transferred to a hydroponic culture system as previously described (Matsuda et al., 2020). Approximately 3 g of fresh roots from three individual 34-day-old soybean plants of each genotype and 2.5 g of polyvinylpolypyrrolidone were homogenized in a mortar with 15 mL of the homogenizing buffer [100 mM phosphate buffer (pH 7.5), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After filtering with Miracloth (Merck, Darmstadt, Germany), the pellet was suspended with 15 mL of a buffer for apoplastic protein extraction [100 mM phosphate buffer (pH 7.5), 1 mM DTT, 1 mM PMSF, 2 M NaCl] and incubated on ice for 1 hour with stirring every 15 min. The suspension was filtered with Miracloth (Merck) and the supernatant was centrifuged (5,800 g, 4 ℃, 10 min). Then, 3 mL of the centrifuged supernatant was applied to a PD-10 column filled with Sephadex G-25 resin (GE Healthcare, Chicago, IL, USA) equilibrated with the homogenizing buffer. Then 3 mL of the homogenizing buffer was applied to the column and the eluate collected and stored at −80 ℃ until further use.

***In vitro* ICHG assay**

Extracted apoplastic crude proteins (10 µg) were mixed with the homogenizing buffer to a volume of 95 µL on ice. After incubating the mixture at 30 ℃ for 3 min, 5 µL of 1 mM malonyldaidzin in methanol was added with gentle tapping and centrifugation. The solution was then incubated at 30 ℃ for 20 min, and the reaction was stopped with 200 µL of 1 % (v /v) formic acid in methanol, followed by centrifugation (20,400 g, 4 ℃,

15 min). The supernatant was filtered with Minisart RC4 (pore size 0.20 µm, diameter 4 mm, Sartorius stedim biotech, Göttingen, Germany) and injected into a high performance liquid chromatography system (LC-10AD, Shimadzu, Kyoto, Japan) with TSKgel ODS-80TM (4.6 × 250 mm, 5 µm, TOSOH Corporation, Tokyo, Japan) to quantitate the daidzein. The LC mobile phase consisted of (A) water and (B) acetonitrile, which both contained 0.3 % (v /v) formic acid, and was eluted isocratically at 36 % of (B) solution for 20 min. The flow rate was 0.8 mL/min and daidzein was detected at 260 nm. Experiments were performed with three technical replicates for all genotypes examined.

Collection of root apoplastic isoflavones and root exudates from soybean seedlings

Apoplastic fractions of the soybean roots were extracted as described previously with some modifications (Li, 2011). One-week-old soybean seedlings were harvested and gently washed twice with deionized water to remove the vermiculite. The roots were cut off, weighed, and then placed into 10 mM sodium phosphate buffer (pH 6.0) in a desiccator and vacuumed on ice. The air pressure in the desiccator was kept under –0.09 MPa for 30 min, then slowly returned to atmospheric pressure. The infiltrated roots were dabbed onto a paper to dry and then placed into plunge barrel of a 10 mL syringe. The syringe barrel was placed in a 50 mL tube and centrifuged at 3,000 g for 15 min at 4 ℃. The collected apoplastic fraction was loaded through a Sep-Pak C18 Plus Short cartridge (Waters). The cartridge was washed with 10 mL of deionized water and eluted with 1.6 mL of methanol. Eluted fractions were dried under nitrogen gas at 50 ℃, reconstituted in

200 µL methanol, and filtered through a 0.45 µm Minisart RC4 filter (Sartorius).

Root exudates from one-week-old soybean seedlings were collected as described previously (Sugiyama et al., 2016). After 24 hours of incubation in nitrogen-deficient mineral nutrient medium at 24 ℃ under 16 h light /8 h dark cycle, the roots were cut off and stored at –80 ℃ for isoflavone extraction.

The extracts were injected into an LC system (ACQUITY H-Class System, Waters, Milford, MA, USA) with ACQUITY UPLC BEH C18 Column (2.1 × 50 mm, 1.7 µm, Waters). The LC mobile phase consisted of (C) water containing 0.1 % (v/v) formic acid and (D) acetonitrile. The gradient program was isocratic at 10 % D, Initial; linear at 0 %–85 % D, 0–15 min; isocratic at 100 % D, 15–16 min; and isocratic at 100 % D, 16–20.5 min. The flow rate was 0.2 mL/min. Isoflavones were detected using a tandem quadrupole MS (Xevo TQD, Waters) and the Multiple Reaction Monitoring (MRM) mode. MRM conditions for this analysis were the same as previously described (Matsuda et al., 2020).

Cultivating and sampling field-grown soybean

Field cultivation of soybean was carried out at the Kyoto University of Advanced Science at Kameoka, Kyoto, Japan (34°99’38”N, 135°55’14”E). Surface-sterilized soybean seeds were sown in pods filled with vermiculite. Seedlings were grown at 23 ℃ under a 16-h-light/8-h-dark cycle for 8 days and then planted in a field on June 25, 2020. The sampling of 7-week-old soybean plants (R1 stage) was conducted on August 5, 2020.

Four soybean plants for each genotype (WT, *ichg-1*, *ichg-2*, and WT-1) were sampled except for WT-2 which was collected for three plants. The upper fully expanded leaves and a few lateral roots were taken from each sample, immediately frozen in dry ice, and then transferred to –80 ℃ until further use. Bulk soil was collected at four different locations at least 20 cm from the plants as previously described (Sugiyama et al., 2014). Whole soybean plants were brought back to the laboratory on ice. The rhizosphere soils for isoflavone analysis were then collected from each plant using brushes, as described previously (Sugiyama et al., 2014). One replicate of Enrei WT rhizosphere samples lacked due to a technical error. Roots were then washed and sonicated in a beaker filled with 100 mL PBS buffer containing 0.02 % Silwet L-77 for 5 min each (Bulgarelli et al., 2012). The washing buffer was then centrifuged at 8,000 g for 10 min at 5 ℃, and the soil was collected for bacterial community analysis. The sonicated roots were rinsed with tap water and then stored at –30 ℃ for DNA extraction.

Cultivating soybeans under nitrogen-sufficient and -deficient conditions

This experiment was conducted according to Yazaki et al. (2021) with minor modifications. One-week-old soybean seedlings were transferred to pots filled with vermiculite. Approximately 4 g of soil, collected from the field at the Kyoto University of Advanced Science, Kameoka, Kyoto, Japan, was placed on the surface of vermiculite as a bacterial inoculation source. Soybean plants were cultivated in a greenhouse at 28 ℃ under natural light conditions at Uji campus, Kyoto University (34°90’87”N,

135°80’22”E) from June 20 to July 6, 2022, and harvested at the V3 stage. Four plants for each genotype were grown in both nitrogen-sufficient and nitrogen-deficient conditions, except for WT-2 under nitrogen-sufficient condition which lacked one replicate due to a technical failure. After removing vermiculite from roots with tap water, nodules were removed, and some of the lateral roots were immediately cut off and frozen with liquid nitrogen for RNA and metabolite extraction. The surface of the remaining roots was washed and sonicated in a PBS buffer with 0.02 % Silwet L-77 for 5 min each and was rinsed with tap water (Bulgarelli et al., 2012). Lateral roots approximately 10 cm from the base were cut off and stored at –30 ℃ for DNA extraction.

RNA-seq and transcriptome analysis

RNA extraction, sequencing, raw-read alignment, normalization, and gene annotation were performed as previously described (Matsuda et al., 2020). The DEGs between the *ICHG* WTs and mutants were determined using the DESeq2 package (Love et al., 2014) in the R environment with a false discovery rate cutoff of 1 %. The transcriptome data set supporting the results of this study is publicly available at the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp) (DRA015007, SSUB023574).

Extraction of isoflavones from plant tissues and soil

The preparation of leaf and root extracts was performed according to Sugiyama et al. (2016). Isoflavone extraction from the rhizosphere and bulk soil was conducted

using a previously described method with some modifications (Sugiyama et al., 2017). In brief, soil samples (approximately 200 mg) were extracted in 3 × 1 mL of methanol at 50 ℃ (10 min each) and centrifuged at 5,000 g for 5 min. The combined supernatant was dried under a nitrogen stream at 50 ℃, re-dissolved in 100 µL methanol, and filtered through a 0.45 µm Minisart RC4 filter (Sartorius). The extracts were analyzed by LC-MS as described above.

Bacterial community analysis using 16S rRNA gene amplicon sequencing

Bacterial community analysis was performed using 16S rRNA gene amplicon sequencing, as previously described with some minor modifications (Nakayasu et al., 2021). Briefly, the V4 region of the 16S rRNA genes was amplified using PCR with the following forward and reverse primers: 515F (5'- acactctttccctacacgacgctcttccgatct-gtgccagcmgccgcggtaa-3') and 806R (5'-gtgactggagttcagacgtgtgctcttccgatct-ggactachvgggtwtctaat-3'), respectively, and sequenced using the MiSeq platform (Illumina). The sequence data were analyzed using QIIME2 version 2021.11 (Bolyen et al., 2019). The 21st to 200th base pair of paired-end sequencing reads were trimmed out and error-corrected amplicon sequence variants (ASVs) were constructed using DADA2 (Callahan et al., 2016) with the q2-dada2 plugin in QIIME2. The α- and β-diversity metrics were estimated with 50,000, 60,000, and 31,500 sequences per rhizosphere and endosphere sample of field-grown soybeans, and endosphere sample of greenhouse-grown soybeans, respectively. Taxonomic assignment of the ASVs was performed using

the Naïve Bayes classifier with SILVA release 138 (Quast et al., 2013; Bokulich et al., 2018). The liner discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) was performed using default parameters to detect differentially abundant bacterial families with an adjusted *p* value < 0.05 for Kruskal-Wallis and an LDA score of >2. The sequence dataset supporting the results of this study is publicly available at DDBJ (https://www.ddbj.nig.ac.jp; DRA013654, DRA015072).

Nodulation test and observation of rhizobial infection threads

B. diazoefficiens USDA110 (MAFF 303215) was obtained from the NARO Genebank (http://www.gene.affrc.go.jp/index_en.php). The DsRed-labeled *B. diazoefficiens* USDA 110 was created as previously described (Nguyen et al., 2020). DsRed-non-labeled and labeled *B. diazoefficiens* USDA 110 was pre-cultured in YEM medium (Estrella et al., 2004). After 7 days, 1 mL of the culture solution was centrifuged (13,000 g, 4 ℃, 2 min) and the pellet was suspended with 1 mL of 10 mM $MgCl_2$. The washing process was repeated twice, and the washed suspension was then diluted to 1 × 10^7 colony forming units (CFU)/1 mL. Ten surface-sterilized soybean seeds were then germinated in a Petri dish with 20 mL sterile water and two pieces of KimWipe (Kimberly-Clark Corp., Irving, TX, USA).

For the nodulation test, all work was staggered by one day for WT-1 and *ichg-1*, Enrei ET, and WT-2 and *ichg-2*. After 5 days, seedlings were transferred into Leonard jars made of two plant boxes (AGC TECHNO GLASS Co., Ltd.) (Ye et al., 2005) and *B.*

588 *diazoefficiens* (1×10^7 CFU) was inoculated onto the seedlings. The soybeans were
589 cultivated in a greenhouse at Uji campus, Kyoto University (34°90’87”N, 135°80’22”E)
590 from November 3–5, 2021 to December 1–3, 2021. When the bottom side of the Leonard
591 jars became empty, 1/2 nitrogen-free medium (Yazaki et al., 2021) was poured into the
592 jar. The room temperature was set at 23 ℃ with supplemental lighting in the morning and
593 evening to create 16-h-light/8-h-dark conditions. After 28 days, 12 soybean plants for
594 each genotype (WT, *ichg-1*, *ichg-2*, and WT-1), and 10 plants for WT-2 were sampled.
595 The soybean shoots were cut and weighed. Their roots were gently washed with tap water
596 and then patted dry. Nodules on the roots were picked using tweezers and counted. The
597 leftover roots and nodules were completely dried at 50 ℃ and then weighed.

598 For the rhizobial infection thread observations, soybean seedlings were
599 transferred to autoclaved plant boxes (AGC TECHNO GLASS Co., Ltd.) five days after
600 germination and the DsRed-labeled *B. diazoefficiens* (1×10^7 CFU) was inoculated onto
601 the seedlings. Each plant box was covered with another sterile plant box to maintain
602 sterility inside the box. After 7 days of incubation at 25 ℃ with 16-h-light/8-h-dark
603 conditions, the roots were washed gently with deionized water. The upper part (<3 cm)
604 of the lateral roots was cut and observed. Fluorescence images of the infection threads
605 were captured using a confocal laser-scanning microscope (FV3000, Olympus, Tokyo,
606 Japan) with a 40 × 0.75 numerical aperture objective. DsRed was monitored by excitation
607 at 561 nm with a 20-mW diode laser and emission at 570–670 nm. Three shots of different
608 infection threads were taken for each genotype.

609

610 **Expression analysis of rhizobium *nod* genes**

611 *B. diazoefficiens* USDA110 was pre-cultured in YEM medium (Estrella et al.,
612 2004) at 28 ℃ with 180 rpm until OD_{600} reached 0.5~1.0. The pre-culture solution was
613 diluted to OD_{600} = 0.1 and dispensed at 4 mL each for four replicates, and added 4 µL of
614 10 mM daidzein, daidzin, and malonyldaidzin standards dissolving in dimethyl sulfoxide,
615 and dimethyl sulfoxide as a mock treatment. After 20 hours of the *nod* gene induction at
616 28 ℃ with 180 rpm, 2 mL of the culture solution was transferred to a 2.0 mL tube and
617 subjected to centrifugation (15,000 g, 4 ℃, 2 min). The pellet was suspended with 1 mL
618 of TRI Reagent® (Molecular Research Center Inc., USA) by pipetting and RNA
619 extraction was performed according to the manufacturer's protocol. The synthesis of
620 cDNA from 200 ng of total RNA was conducted by using ReverTra Ace® qPCR RT
621 Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan). For qPCR, 1 µL of
622 synthesized cDNA (×1/4 dilution) was added as a template to THUNDERBIRD®
623 SYBR® qPCR Mix (Toyobo Co., Ltd., Japan). The qRT-PCR reactions were performed
624 and primer sets for amplifying *nod* genes of *B. diazoefficiens* USDA 110 were designed
625 according to Sugawara and Sadowsky (2013) and Ahmad et al. (2021).

626

627 **Statistical analysis**

628 All boxplots in this study were created in the R environment (v. 4.1.2; R Core
629 Team, 2021) using the ggplot2 package and the outliers were identified with a default

parameter (1.5 × interquartile range). The statistical analysis of plant transcriptomic data was conducted using the adonis function in the vegan R package. Since the F_2 progenies of two independent *ichg* mutants screened from the mutant library are likely to have different genetic backgrounds, the Student's two-tailed t-test was used for calculating statistically significant differences (*$p < 0.05$) between the missense mutant-derived the *ichg-1* and WT-1 and the nonsense mutant-derived the *ichg-2* and WT-2, respectively. In the nitrogen-controlled experiment, the Student's two-tailed t-test was used for comparing N+ samples with N- samples in the same genotypes (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Tukey's HSD test was used for the analysis of isoflavone contents under N+ and N- conditions among Enrei WT, the *ichg* mutant and WT allele from missense and nonsense mutants, respectively ($p < 0.05$). The statistical analysis of bacterial communities was conducted using unweighted and weighted UniFrac-based PERMANOVA in QIIME2. Dunnett's test was used for statistical analysis on daidzein, daidzin, and malonyldaidzin-treated samples compared to the DMSO-treated sample (*$p < 0.05$).

Supplementary materials

Supplemental Figure S1. Comparison of the bacterial communities from field-grown *ichg* mutants and WTs. (A) Rarefaction curves for the number of observed sequence variants in the endosphere and rhizosphere and bulk soil samples. (B) Unweighted and weighted UniFrac-based PCoA of the endosphere, rhizosphere, and bulk soil bacterial communities

(endosphere and rhizosphere, n = 19; bulk soil, n = 4). Diamonds, squares, and circles indicate the bulk soil, rhizosphere, and endosphere, respectively. (B) Relative abundance of *Comamonadaceae* in the endosphere and rhizosphere bacterial communities (WT-2, n = 3; the other genotypes, n = 4). The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. Student's two-tailed t-test was used for statistical analysis between the *ichg* mutant and WT allele from missense and nonsense mutants, respectively ($p < 0.05$). ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; PCoA, principal coordinate analysis; WT, wild-type.

Supplemental Figure S2. mRNA levels of known genes up-regulated under nitrogen-deficient (N-) conditions in roots of *ichg* mutants and WTs (WT-2 N+, n = 3; others, n = 4). The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. Student's two-tailed t-test was used for statistical analysis between nitrogen-sufficient (N+) and N- conditions in the same genotypes ($p < 0.05$). CHR, chalcone reductase; CHS, chalcone synthase; HID, 2-hydroxyisoflavanone dehydratase; IFS, isoflavone synthase; TPM, transcripts per million; WT, wild-type.

Supplemental Figure S3. Comparison of the root bacterial communities from *ichg* mutants and WTs cultivated under nitrogen-sufficient (N+) and -deficient (N-) conditions (WT-2 N+, n = 3; others, n = 4). (A) Rarefaction curves for the number of observed sequence variants. (B) Unweighted and weighted UniFrac-based PCoA of the endosphere

672 bacterial communities. Circles and inverted triangles indicate samples cultivated under
673 N+ and N-, respectively.
674
675 Supplemental Figure S4. Fluorescent images of the DsRed-labeled rhizobial infection
676 threads on soybean roots. *Bradyrhizobium diazoefficiens* USDA 110 was inoculated onto
677 5-day-old soybean seedlings under sterile conditions and infection threads on the roots
678 were observed after 7 days. Three representative shots of different infection threads for
679 each genotype are shown. ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; WT,
680 wild-type.
681
682 Supplemental Figure S5. mRNA levels of transporter genes in roots of *ichg* mutants and
683 WTs cultivated in (A) field and (B) greenhouse. These transporter genes were co-
684 expressed diurnally ($r > 0.8$) with isoflavone biosynthetic genes (Matsuda et al., 2020).
685 *Glyma.01G026200*, a multidrug and toxin extrusion transporter; *Glyma.10G019000*, a
686 ATP-binding cassette (ABC) transporter C subfamily; *Glyma.13G361900* and
687 *Glyma.15G011900*, ABC transporter G subfamily. The individual black dots indicate raw
688 data points. The outliers were identified using the 1.5 × interquartile range rule. (A)
689 Student's two-tailed t-test was used for statistical analysis between nitrogen-sufficient
690 (N+) and nitrogen-deficient (N-) conditions in the same genotypes (WT-2, $n = 3$; others,
691 $n = 4$) ($p < 0.05$). (B) Tukey's HSD test was used for statistical analysis among Enrei WT,
692 the *ichg* mutant and WT allele from missense and nonsense mutants, respectively (WT-2

693 N+, n = 3; others, n = 4) ($p < 0.05$). ICHG, isoflavone conjugate-hydrolyzing β-
694 glucosidase; TPM, transcripts per million; WT, wild-type.
695
696 Supplemental Figure S6. Relative expression levels of *nod* genes of *Bradyrhizobium*
697 *diazoefficiens* USDA110 inducted by isoflavone standard chemicals. Dunnett's test was
698 used for statistical analysis on daidzein, daidzin, and malonyldaidzin-treated samples
699 compared to DMSO-treated sample ($p < 0.05$). DMSO, dimethyl sulfoxide.
700
701 Supplemental Table S1. PERMANOVA results for the first two PCs in the principal
702 component analysis of transcriptome of field-grown *ichg* mutants and wild-types (WTs).
703
704 Supplemental Table S2. Number of Differentially expressed genes (DEGs) in the *ichg*
705 mutants when compared with the *ICHG* WTs (false discovery rate < 0.01).
706
707 Supplemental Table S3. DEGs between field-grown WT-1 and *ichg-1* (false discovery
708 rate < 0.01).
709
710 Supplemental Table S4. DEGs between field-grown WT-2 and *ichg-2* (false discovery
711 rate < 0.01).
712
713 Supplemental Table S5. Unweighted and weighted UniFrac-based PERMANOVA results

714 for the endosphere, rhizosphere, and bulk soil samples.

715

716 Supplemental Table S6. Unweighted and weighted UniFrac-based PERMANOVA results
717 for the endosphere bacterial communities of each sample.

718

719 Supplemental Table S7. Unweighted and weighted UniFrac-based PERMANOVA results
720 for the rhizosphere bacterial communities of each sample.

721

722 Supplemental Table S8. Number of enriched and depleted bacterial families in the *ichg*
723 mutants when compared with *ICHG* WTs using LEfSe method (LDA score >2.0).

724

725 Supplemental Table S9. Enriched and depleted endosphere bacterial families in *ichg-1*
726 and *ichg-2* in comparison with WT-1 and WT-2 using LEfSe method (LDA score >2.0).

727

728 Supplemental Table S10. Enriched and depleted rhizosphere bacterial families in *ichg-1*
729 and *ichg-2* in comparison with WT-1 and WT-2 using LEfSe method (LDA score >2.0).

730

731 Supplemental Table S11. PERMANOVA results for the first two PCs in the principal
732 component analysis of the root transcriptome of *ichg* mutants and WTs cultivated under
733 nitrogen-sufficient (N+) and nitrogen-deficient (N-) conditions.

734

735 Supplemental Table S12. DEGs between N+ and N- conditions (false discovery rate <
736 0.01).
737
738 Supplemental Table S13. Unweighted and weighted UniFrac-based PERMANOVA
739 results for endosphere samples cultivated under N+ and N- conditions.
740
741 Supplemental Table S14. Unweighted and weighted UniFrac-based PERMANOVA
742 results for the endosphere bacterial communities under N+ condition.
743
744 Supplemental Table S15. Unweighted and weighted UniFrac-based PERMANOVA
745 results for the endosphere bacterial communities under N- condition.
746
747 Supplemental Table S16 Enriched endosphere bacterial genera under N+ condition using
748 LEfSe method (LDA score >2.0).
749
750 Supplemental Table S17 Enriched endosphere bacterial genera under N- condition using
751 LEfSe method (LDA score >2.0).
752
753 Supplemental Table S18. Enriched and depleted endosphere bacterial genera in *ichg-1*
754 and *ichg-2* in comparison with WT-1 and WT-2 using LEfSe method under N+ conditions
755 (LDA score >2.0).

Supplemental Table S19. Enriched and depleted endosphere bacterial genera in *ichg-1* and *ichg-2* in comparison with WT-1 and WT-2 using LEfSe method under N- conditions (LDA score >2.0).

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Figure legends

Figure 1. Proposed isoflavone supply routes from the cytosol to the rhizosphere. The apoplast includes the cell walls and intercellular space. ABC, ATP-binding cassette transporter; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; MaT, malonyl transferase; MATE, multidrug and toxic compound extrusion transporter; PM, plasma membrane; UGT, UDP-glucuronosyltransferase.

Figure 2. Development of *ichg* mutants from a soybean high-density mutant library. (A) Gene mutations and amino acid replacements in the *ICHG* gene of *ichg-1* and *ichg-2* (*Glyma.12G053800*, genome sequence 6,088 bp from start to stop codon, peptide sequence 514 aa). Gray squares show exons of *ICHG*. In *ichg-1*, the 420th glutamic acid is replaced by lysine (peptide sequence 514 aa). In *ichg-2*, the 232nd glutamine is replaced by a stop codon (peptide sequence 231 aa). (B) Scheme for acquiring homozygous *ichg* mutants and WT alleles. Missense and nonsense *ichg* mutants from the soybean (cultivar Enrei) mutant library were backcrossed with Enrei WT, and F_2 progenies were obtained through the self-pollination of the F_1 plants. (C) ICHG activity of the apoplastic crude proteins extracted from the roots of WT soybean and two homozygous *ichg* mutants and their WT alleles. The individual black dots indicate raw data points. Bar graphs show the mean of three technical replicates. ICHG activity of the apoplastic crude proteins extracted from *ichg-1* and *ichg-2* was below the detection limit. ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; N.D., not detected; WT, wild-type.

Figure 3. Individual and total contents of the major isoflavones in the root apoplastic fractions (A, B) and whole roots (C, D) of 1-week-old *ichg* mutants and WTs (n = 4). (A, C) The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. (B, D) The sum of daidzein, genistein, their glucoside, and malonylglucoside contents. Bar graphs show the mean of the biological replicates and circles indicate raw data points for each replicate. The Student's two-tailed t-test was

used for statistical analysis between the *ichg* mutant and WT allele from the missense and nonsense mutants, respectively ($p < 0.05$). AF, apoplastic fraction; DW, dry weight; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; WT, wild-type.

Figure 4. Individual and total contents of the major isoflavones in the root exudates (A, B) and roots (C, D) of 1-week-old *ichg* mutants and WTs after cultured hydroponically for 24 hours (WT-1, n = 4; others, n = 5). (A, C) The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. (B, D) The sum of daidzein, genistein, their glucoside, and malonylglucoside contents. Bar graphs show the mean of the biological replicates and circles indicate raw data points for each replicate. The Student's two-tailed t-test was used for statistical analysis between the *ichg* mutant and WT allele from the missense and nonsense mutants, respectively ($p < 0.05$). DW, dry weight; FW, fresh weight; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; WT, wild-type.

Figure 5. Transcriptome comparison of field-grown *ichg* mutants and WTs (WT-2, n = 3; others, n = 4). (A) mRNA levels of *ICHG* in the leaves and roots. The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. Student's two-tailed t-test was used for statistical analysis between the *ichg* mutant and WT allele from the missense and nonsense mutants, respectively ($p < 0.05$). (B) Scatter plot of the first two PCs in the principal component analysis of the leaf and

819 root transcriptomes. Circles and triangles indicate the *ICHG* WTs and *ichg* mutants,
820 respectively. ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; PC, principal
821 component; TPM, transcripts per million; WT, wild-type.
822
823 Figure 6. Individual and total contents of the major isoflavones in the leaves (A, B), roots
824 (C, D), and rhizosphere soil (E, F) collected from field-grown *ichg* mutants and WTs
825 (WT-2, n = 3; others, n = 4). Rhizosphere samples of Enrei WT lacked one replicate due
826 to a technical error. (A, C, E) The individual black dots indicate raw data points. The
827 outliers were identified using the 1.5 × interquartile range rule. (B, D, F) The sum of
828 daidzein, genistein, their glucoside, and malonylglucoside contents. Bar graphs show the
829 mean of biological replicates and the circles indicate raw data points for each replicate.
830 Student's two-tailed t-test was used for statistical analysis between the *ichg* mutant and
831 WT allele from missense and nonsense mutants, respectively ($p < 0.05$). DW, dry weight;
832 ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; N.D., not detected; WT, wild-
833 type.
834
835
836 Figure 7. Unweighted and weighted UniFrac-based PCoA of the endosphere (A) and
837 rhizosphere (B) bacterial communities from field-grown *ichg* mutants and WTs (WT-2,
838 n = 3; others, n = 4). Circles and triangles indicate *ICHG* WTs and mutants, respectively.
839 ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; PCoA, principal coordinate

840 analysis; WT, wild-type.

841

842 Figure 8. Analysis of *ichg* mutants and WTs cultivated under nitrogen-sufficient (N+) and
843 -deficient (N-) conditions (WT-2 N+, n = 3; others, n = 4). (A) Scatter plot of the first
844 two PCs in the principal component analysis of the transcriptome of *ichg* mutants and
845 WTs. Circles and inverted triangles indicate samples cultivated under N+ and N-,
846 respectively. (B) mRNA levels of *ICHG* in roots. The individual black dots indicate raw
847 data points. The outliers were identified using the 1.5 × interquartile range rule. Student's
848 two-tailed t-test was used for statistical analysis between N+ and N- conditions in the
849 same genotypes ($p < 0.05$). (C) Contents of major isoflavones in roots. The individual
850 black dots indicate raw data points. The outliers were identified using the 1.5 ×
851 interquartile range rule. Tukey's HSD test was used for statistical analysis among Enrei
852 WT, the *ichg* mutant and WT allele from missense and nonsense mutants, respectively (p
853 < 0.05). (D) Relative abundance of unannotated genus of *Comamonadaceae* and
854 *Bradyrhizobium*. The individual black dots indicate raw data points. The outliers were
855 identified using the 1.5 × interquartile range rule. For *Bradyrhizobium*, one replicate in
856 Enrei WT N- is excluded in the boxplot due to its extremely high abundance compared
857 to other samples. Student's two-tailed t-test was used for statistical analysis between N+
858 and N- conditions in the same genotypes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). DW,
859 dry weight; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; PC, principal
860 component; TPM, transcripts per million; WT, wild-type.

Figure 9. Nodulation assessment of *ichg* mutants and WTs (WT-2, n = 10; others, n = 12). (A) fresh weight of the shoots. (B) dry weight of the roots. (C, D) Nodule number (C) and dry weight (D). The individual black dots indicate raw data points. The outliers were identified using the 1.5 × interquartile range rule. Student's two-tailed t-test was used for statistical analysis between the *ichg* mutant and WT allele from missense and nonsense mutants, respectively ($p < 0.05$). DW, dry weight; FW, fresh weight; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase; WT, wild-type.

Figure 10. Heatmaps showing the fold change of isoflavone glycoside and aglycone contents in roots (yellow box), the apoplastic fraction (light blue box), and rhizosphere soil (brown box) of *ichg* mutants compared with those of WTs. The color of each cell corresponds to the $\log_2$(fold change) of the mean of isoflavone aglycone (the sum of daidzein (R_1 = H) and genistein (R_1 = OH)) or glycoside (the sum of their glucosides (R_2 = H) and malonylglucosides (R_2 = $COCH_2COOH$)) contents in *ichg-1* compared to WT-1, or *ichg-2* compared to WT-2. Red and blue colors indicate the increase and decrease in *ichg* mutants against the WTs, respectively.

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