Flavonoids and saponins in plant rhizospheres: roles, dynamics, and the potential for agriculture

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Abstract

Plants are in constant interaction with a myriad of soil microorganisms in the rhizosphere, an area of soil in close contact with plant roots. Recent research has highlighted the importance of plant specialized metabolites (PSMs) in shaping and modulating the rhizosphere microbiota; however, the molecular mechanisms underlying the establishment and function of the microbiota mostly remain unaddressed. Flavonoids and saponins are a group of PSMs whose biosynthetic pathways have largely been revealed. Although these PSMs are abundantly secreted into the rhizosphere and exert various functions, the secretion mechanisms have not been clarified. This review summarizes the roles of flavonoids and saponins in the rhizosphere with a special focus on interactions between plants and the rhizosphere microbiota. Furthermore, this review introduces recent advancements in the dynamics of these metabolites in the rhizosphere and indicates potential applications of PSMs for crop production and discusses perspectives in this emerging research field.

Keywords

flavonoid, holobiont, rhizosphere, saponin, soybean

Introduction

Plants produce a diverse array of low molecular weight compounds, the number of which exceeds 200,000 (Fernie 2007; Afendi et al. 2012). A large portion of these metabolites are not necessarily essential for growth and development but often have biological activities such as protection against biotic and abiotic stress and modulation of interactions with other organisms. These metabolites are called plant specialized metabolites (PSMs), and it is thought that plants acquired the ability to biosynthesize these metabolites during evolution for adaptation to the ecosystem (Kim and Buell 2015; Lichman et al. 2020).

In nature, plants are in constant interaction with a myriad of microorganisms ranging from pathogens, commensals, and beneficial microbes. It is believed that plants have evolved to benefit from beneficial microorganisms while protecting themselves from pathogens; however, how to distinguish these microbes at the molecular level remains unclear. How do plants engage with beneficial microorganisms while at the same time restricting pathogens is among the top unanswered questions about molecular plant-microbe interactions (Harris et al. 2020), and answering this question is necessary for the utilization of these microbes for sustainable crop production (Finkel et al. 2017; Zhang et al. 2017). The rhizosphere, the region of soil in proximity to plant roots, is a hotspot for these interactions, and it harbors unique repositories of microbes and metabolites (Hartmann et al. 2008). Plant roots exude a substantial amount (up to 40%) of photosynthesis-derived carbons into the rhizosphere (Lynch and Whipps 1990; Badri and Vivanco 2009; Haichar et al. 2016). The majority of them are primary metabolites including sugars, amino acids, and organic acids, which nourish rhizosphere microbes (Canarini et al. 2019). In addition to the primary metabolites, plant roots secrete PSMs

that have important roles in the interactions between plants and soil microbes.

Root-derived metabolites, along with the physicochemical properties of soil and environment factors, influence soil microbial communities and affect the formation of rhizosphere microbiota characterized by abundant and active microbial communities having reduced diversity, as compared with bulk soil (Wang et al. 2020; Vieira et al. 2020). Recent evidence supports the importance of the rhizosphere microbiota in plant growth, immunity, and fitness, emphasizing the potential for utilization in crop production (Canto et al. 2020; Compant et al. 2019). The involvement of PSMs in shaping the root/rhizosphere microbiota was demonstrated using plant mutants disrupted in a particular biosynthetic pathway and has been summarized in recent reviews (Pascale et al. 2020; Jacoby et al. 2021; Pang et al. 2021). In addition to the loss-of-function approach, we have employed an artificial rhizosphere treatment method to reveal the functions of flavonoids and saponins in shaping the rhizosphere microbiota (Okutani et al. 2020; Fujimatsu et al. 2020) (Nakayasu et al. 2021) (Figure 1). In this review, we focus on two types of PSMs—flavonoids and saponins—that are abundantly secreted from roots to summarize their functions and dynamics in the rhizosphere and highlight future challenges in harnessing rhizosphere microbiota for plant robustness and sustainable crop production.

Flavonoids

Synthesis and accumulation

Flavonoids are phenolic compounds comprising more than 8,000 distinct molecules (Pietta 2000). Flavonoids confer protection from UV-B radiation, pathogen, and herbivores, attract pollinators, regulate auxin transport, and modulate reactive oxygen

87 species and fertility in plants (Ferreyra et al. 2012). Additionally, flavonoids are secreted
88 from roots and exert diverse roles in the rhizosphere (see below). Flavonoids in plants are
89 biosynthesized through the phenylpropanoid and the acetate-malonate pathways to form
90 molecules composed of 15 carbon atoms arranged as C_6-C_3-C_6. Chalcone synthase (CHS)
91 catalyzes the first step in flavonoid biosynthesis using *p*-coumaroyl-CoA and malonyl-
92 CoA as substrates (Yonekura-Sakakibara et al. 2019). Based on the oxidation and
93 substitution of the C (C_3) ring, flavonoids are further categorized into the subgroups,
94 including anthocyanins, flavonols, flavanones, flavones, flavanols, isoflavones,
95 chalcones, catechins, and aurones (Panche et al. 2016). Isoflavones are predominantly
96 found in legume plants (Figure 2). Daidzein and genistein in soybean are biosynthesized
97 from liquiritigenin and naringenin, respectively, by isoflavone synthase (IFS), a P450
98 protein, to form 2-hydroxyisoflavanone (Akashi et al. 1999; Jung et al. 2000), followed
99 by dehydration to produce daidzein and genistein either spontaneously or via 2-
100 hydroxyisoflavanone dehydratase (HID) (Akashi et al. 2005). Glycitein is biosynthesized
101 from liquiritigenin by flavonoid 6-hydroxylase to form 6-hydroxyliquiritigenin (Latunde-
102 Dada et al. 2001). 6-hydroxyliquiritigenin is then converted to 6-hydroxydaidzein by IFS
103 and HID and subsequently converted to glycitein by isoflavone *O*-methyltransferase
104 (Uchida et al. 2020).

105 Although direct biochemical evidence is lacking, most flavonoids in plant cells
106 are assumed to be accumulated in vacuoles in the form of glucosides. Vacuolar
107 sequestration of flavonoids involves transporters, glutathione S-transferase, and vesicle
108 trafficking (Zhao 2015). Both ATP-binding cassette (ABC) transporters and multidrug
109 and toxin extrusion (MATE) transporters have been identified as being involved in the
110 vacuolar sequestration of flavonoids (Zhao 2015). Most of the research on vacuolar

111 sequestration has analyzed the sequestration of anthocyanins and proanthocyanidins
112 (Zhao 2015; Shitan and Yazaki 2020); however, biochemical transport analysis using
113 yeast membrane vesicles revealed that AtABCC2 of *Arabidopsis thaliana* transports
114 flavonoid glucosides such as luteolin 7-*O*-glucoside and apigenin 7-*O*-glucoside, in
115 addition to anthocyanins (Behrens et al. 2019), and MtMATE2 of *Medicago truncatula*
116 transports apigenin 7-*O*-glucoside, apigenin 7-*O*-glucoside malonate, kaempferol 7-*O*-
117 glucoside, and kaempferol 7-*O*-glucoside malonate, in addition to anthocyanins (Zhao et
118 al. 2011).
119
120 *Secretion into the rhizosphere*
121 Root exudates and root border cells are the primary sources of rhizosphere flavonoids
122 (Sasse et al. 2018; Hassan and Mathesius 2012). ABC transporters have been suggested
123 to mediate the secretion of flavonoids into the rhizosphere. A biochemical transport assay
124 using plasma membrane vesicles of soybean revealed the involvement of ABC
125 transporters in the secretion of genistein, an isoflavone acting as a signal for nod gene
126 expression in rhizobia (Sugiyama et al. 2007). Tobacco BY-2 cells expressing
127 MtABCG10 showed the efflux of isoliquiritigenin from cells (Biala et al. 2017).
128 Soybean is a suitable model plant to study flavonoid secretion because of its
129 relatively large leaves and roots and the importance of secreted flavonoids in its
130 interactions with rhizosphere microbes (Kosslak et al. 1987; Sugiyama 2019; Okutani et
131 al. 2020). Three types of isoflavone aglycones—daidzein, genistein, and glycitein—are
132 biosynthesized in the cytosol of soybean, and their glucosides and malonylglucosides are
133 presumably accumulated in the vacuoles (Figure 2A). These aglycones are also secreted
134 from roots and found in root exudates (Pueppke et al. 1998). In addition to the ATP-

dependent active transport of isoflavone aglycones, secretion of isoflavone glucosides stored in vacuoles into the apoplast has also been proposed (Suzuki et al. 2006; Sugiyama 2019). Secreted isoflavone glucosides are hydrolyzed to aglycones by isoflavone conjugate hydrolyzing β-glucosidase (ICHG) (Suzuki et al. 2006).

The secretion of isoflavones from soybean roots has been analyzed both in hydroponic conditions and in the field. Both daidzein and genistein induce *nod* genes of the rhizobia, leading to a symbiosis for biological nitrogen fixation (Kosslak et al. 1987; Pueppke et al. 1998). Under nitrogen deficiency, the secretion of these isoflavones is increased approximately 10-fold in hydroponic conditions (Sugiyama et al. 2016) (Figure 3). Daidzein is the predominant isoflavone in root exudates throughout the growth stages; a higher amount of daidzein is secreted during the vegetative stages than the reproductive stages, and the secretion of malonyldaidzin and daidzin is increased during the reproductive stages (Sugiyama et al. 2016). The amount of daidzein and genistein in root exudates is stable during the day. However, genes for transcription factors and biosynthesis of isoflavone metabolism show diurnal regulation with increased expression during the daytime (Matsuda et al. 2020). GmMYB176, a transcription factor of isoflavone biosynthesis highly expressed in roots, induces isoflavone biosynthetic genes from dawn to noon, followed by the induction of isoflavone biosynthetic genes at noon, and a slight increase of daidzein aglycone in roots in the afternoon (Matsuda et al. 2020). Conceivably, the secretion pathway from vacuolar glucosides to apoplast would be induced during the nighttime when the expression of *ICHG* is increased to maintain the daidzein level in root exudates.

In field-grown soybean, the secretion of daidzein is also higher in the early vegetative stages than in the reproductive stages, but the amount is increased up to

10,000-fold, compared with hydroponic conditions (Toyofuku et al. 2021) (Figure 3). The increase of secretion is possibly due to the presence of microbes in the field; however, soil particles in the field may influence the root morphology and the rate of isoflavone secretion because the particle size and chemistry of growth substrates such as sand, clay, and glass beads, affect root morphology and exudation in *Brachypodium distachyon* (Sasse et al. 2020). It remains unclear whether both pathways of isoflavone secretion, i.e. active transport of isoflavone aglycones and secretion of vacuole-stored isoflavone glucosides, operate simultaneously or if one operates conditionally. Although higher daidzein secretion is suggested during the early vegetative stages, the rhizosphere isoflavone contents are slightly higher in the reproductive stage due to relative stability of the compounds in the soil (Sugiyama et al. 2017). It is of particular importance to analyze the microbial communities and physicochemical properties of soils together with degradation kinetics since the stability of flavonoids depends on the soil (Sugiyama and Yazaki 2014).

Roles in the rhizosphere

Flavonoids display a broad range of biological activities not only in plants but in animals and microbes. The biological activities in humans include antioxidative activity, free radical scavenging capacity, anti-inflammatory capacity, and anticancer activities (Gorniak et al. 2019), and flavonoids are widely used as phytomedicines. Flavonoids inhibit a range of root pathogens in the rhizosphere (Hassan and Mathesius 2012; Mierziak et al. 2014) because of their antipathogenic properties, such as the ability to disrupt membrane integrity (Weinstein and Albersheim 1983; Wu et al. 2019) and inhibit DNA gyrase (Wu et al. 2013). These defensive flavonoids can be exuded into the soil

183 either constitutively or inductively; for example, an increased level of glyceollin I, a
184 phytoalexin of soybean, is found in the root exudate upon exposure to pathogens and non-
185 symbiotic rhizobia (Schmidt et al. 1992; Lozovaya et al. 2004), isoflavones in the root
186 exudates of white lupin are increased upon treatments with various elicitors (Gagnon and
187 Ibrahim 1997). Other roles of flavonoids in the rhizosphere include mediation of
188 allelopathy, chelation, and reduction of metals in soil (Hassan and Mathesius 2012; Cesco
189 et al. 2012; Weston and Mathesius 2013). The following sections highlight the functions
190 of flavonoids as regulators of the rhizosphere microbiome.
191
192 *Roles as chemoattractants*
193 In addition to functioning as a nutrient source for rhizosphere microorganisms, plant
194 metabolites (particularly PSMs) exert both attractive and repellent effects on soil
195 microbes and shape the rhizosphere microbiota (Pascale et al. 2020; Jacoby et al. 2021).
196 Flavonoids have been thought of as an attractant for the rhizobia, as the chemical signals
197 from plant roots to symbiotic rhizobia have been determined to be flavonoids. Several
198 reports have showen a chemotactic activity of *nod* gene-inducing flavonoids, including
199 luteolin, 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and 4,4'-dihydroxy-2-
200 methoxychalcone for *Ensifer meliloti* (Caetanoanolles et al. 1988; Dharmatilake and
201 Bauer 1992) and also apigenin and luteolin for *Rhizobium leguminosarum* (Aguilar et al.
202 1988); however, a recent comprehensive analysis revealed that *E. meliloti* did not show a
203 chemotactic response to flavonoids such as hyperoside, luteolin, luteolin-7-*O*-glucoside,
204 quercetin, and chrysoeriol in alfalfa seed exudates (Compton et al. 2020). Amino acids
205 and quaternary ammonium compounds, which are 10-fold more abundant than flavonoids
206 in alfalfa root exudates, are the primary chemoattractants of *E. meliloti* (Compton et al.

2020). Bacterial chemoreceptors for these metabolites have also been identified in *E. meliloti* (Webb et al. 2014; Webb et al. 2017). In the case of soybean-rhizobia interactions, *Bradyrhizobium japonicum* is most attracted to succinate, glutamate, and malonate and is not attracted to luteolin, daidzein, or genistein (Barbour et al. 1991). *Bradyrhizobium japonicum* is also attracted to cinnamic acid and hydroxycinnamic acids, such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapinic acid (Kape et al. 1991). Together, these studies do not favor the contribution of flavonoids in the recruitment of rhizobia to the proximity of plant roots but indicate the contribution of amino acids, dicarboxylic acids, and quaternary ammonium compounds. Future research is needed to highlight the functions of these chemoattractants in the plant rhizosphere with consideration for the secretion, degradation, and distribution within the context of the soil community where multiple interactions occur.

Roles as nod gene inducers

Once attracted to the vicinity of the root surface, chemical signal exchange between legume plants and the rhizobia occurs (Janczarek et al. 2015; Liu and Murray 2016). Luteolin and 7,4'-dihydroxyflavone were the first signaling compounds discovered in alfalfa and white clover, respectively, using a *nod-lacZ* expression system, which was also used to identify other signaling flavonoids in other legume species, such as daidzein and genistein in soybean (Peters et al. 1986; Redmond et al. 1986; Kosslak et al. 1987; Kape et al. 1991). Different classes of flavonoids, including flavones, flavonols, flavanones, isoflavones, and chalcones, have since been identified as plant signals for induction of *nod* genes in the rhizobia in addition to non-flavonoid metabolites (Janczarek et al. 2015; Liu and Murray 2016). Most of these flavonoids induce *nod* genes at low micromolar

231 concentrations within the range of the rhizospheric concentrations, at least for daidzein
232 (Sugiyama et al. 2017; Toyofuku et al. 2021). The specificity of flavonoid profiles in each
233 legume plant and the specific perception of flavonoids by NodD, a LysR-type
234 transcription regulator, in the rhizobia are responsible for the first level of host specificity
235 for legume-rhizobia symbiosis. Upon perception of the signal, the rhizobia synthesize and
236 secrete lipo-chitooligosaccharide Nod factors (NFs) to be recognized by receptors on the
237 root surface (Gourion et al. 2015; Buhian and Bensmihen 2018). Genetic signaling
238 pathways governing NF detection and nodule organogenesis are now quite well
239 understood in the model legume plants, *Medicago truncatula* and *Lotus japonicus* (Roy
240 et al. 2020). In addition to NFs, type III secretion systems, together with exported proteins,
241 are induced by genistein in *Sinorhizobium fredii* USDA257, a symbiont of soybean and
242 other legume plants (Krishnan et al. 2003), and *B. elkanii* SEMIA587 (de Campos et al.
243 2011). Genistein also induces the expression of resistance-nodulation-division (RND)
244 efflux pumps in *B. japonicum* (Takeshima et al. 2013). A *B. japonicum* mutant deficient
245 in this efflux pump is sensitive to genistein (but not to daidzein) and shows reduced
246 nodulation and nitrogen fixation when this mutant is inoculated in soybean roots. The
247 expression of this efflux pump is negatively regulated by a TerR-like regulator (BdtR)
248 (Han et al. 2020). Mutation of this regulator results in higher extracellular genistein levels
249 and decreased susceptibility to genistein because of induction of the efflux pump. In
250 contrast, the induction of *nod* genes is reduced in the mutant. These results suggest that
251 the rhizobia maintain intracellular genistein homeostasis to induce *nod* genes for
252 nodulation while the toxic effect of this isoflavone is alleviated.
253
254 *Roles in modulating the microbiota*

Flavonoids in the rhizosphere impact the microbiota as well as the rhizobia. The application of pure flavonoid compounds modifies both bacterial and fungal communities in soil. Daidzein and genistein applied to the soil resulted in different microbial community structures, as revealed by phospholipid fatty acid profiling (Guo et al. 2011). The treatment of soils with 7,4′-dihydroxyflavone, a *nod* gene-inducing flavonoid of alfalfa roots, modified bacterial communities at a concentration found in root exudates, with an increase in Acidobacteria, suggesting multiple functions of this flavonoid in the rhizosphere beyond the establishment of symbiosis (Szoboszlay et al. 2016). Both bacterial and fungal communities of the peanut rhizosphere were modified when luteolin was continuously applied to the soil (Wang et al. 2018). The growth and nodule formation of peanuts treated with luteolin were reduced, suggesting the inhibitory effects of luteolin in continuous mono-cropped peanut systems, leading to reduced productivity. Daidzein also modifies bacterial communities in soil (Okutani et al. 2020). The relative abundance of Comamonadaceae was increased in a concentration-dependent manner, and the bacterial communities became more similar to the rhizosphere of soybean grown in fields rather than bulk soil. The rhizosphere bacterial communities of soybean hairy roots silenced with the *IFS* gene showed a slight change, depending on the gene silencing and hairy root transformation (White et al. 2017). Comamonadaceae were not reduced in the IFS-silenced hairy root rhizosphere, suggesting the involvement of multiple metabolites in modulating the rhizosphere microbiota. In maize, flavones such as apigenin and luteolin are secreted from the roots and promote the enrichment of Oxalobacteraceae in the rhizosphere (Yu et al. 2021). Oxalobacteraceae isolates belonging to the genus *Massilia* improve the growth of maize under nitrogen-deficient conditions via alteration of root development, suggesting a network of root architecture and the microbial taxa in

279 the rhizosphere, resulting in improved plant growth under nutrient deficiency.

280

281 **Saponins**

282 *Synthesis and accumulation*

283 Saponins are a group of PSMs widely distributed in higher plants (Vincken et al. 2007).
284 They contain an aglycone hydrophobic backbone bound to hydrophilic saccharides such
285 as glycosides, resulting in amphiphilicity and the formation of a soap-like foam when
286 agitated in water. The name saponin is derived from the Latin word 'sapo', meaning soap,
287 and they are typically subdivided into triterpenoid and steroid glycosides based on the
288 carbon skeletons (Vincken et al. 2007). Both types of compounds are biosynthesized from
289 a common precursor, 2,3-oxidosqualene, via multiple reactions such as cyclization,
290 oxidation, and glycosylation. In the plant kingdom, dicotyledonous plants mainly
291 accumulate triterpenoid saponins, while monocotyledonous plants mainly synthesize
292 steroidal saponins (with some exceptions) (Sparg et al. 2004; Moses et al. 2014).

293 The triterpenoid and steroidal aglycone backbones are synthesized from
294 isopentenyl diphosphate units derived from the mevalonate pathway. Condensation of
295 two farnesyl diphosphate by squalene synthase (SQS) generates squalene, which is then
296 epoxidized to 2,3-oxidosqualene catalyzed by squalene epoxidase (SQE). Oxidosqualene
297 cyclases (OSCs) catalyze the cyclization of 2,3-oxidosqualene, and they are positioned at
298 a key metabolic branch point between primary metabolism for plant sterols (phytosterols)
299 and brassinosteroid hormones and specialized metabolism for triterpenoids. 2,3-
300 Oxidosqualene can be cyclized into a diverse range of compounds with triterpene
301 backbones, including dammaranes, tirucallanes, lupanes, hopanes, oleananes,
302 taraxasteranes, ursanes, lanostanes, and cucurbitanes (Vincken et al. 2007). OSCs

303 catalyzing the cyclization of 2,3-oxidosqualene are either specific or multifunctional,
304 leading to either a single product or multiple products from a single reaction (Moses et al.
305 2014). Following cyclization, triterpene aglycones are oxidized by cytochrome P450 and
306 further modified by an array of transferases such as UDP-dependent glycosyltransferases
307 (UGTs), acyltransferases, malonyltransferases, and methyltransferases (Thimmappa et al.
308 2014; Seki et al. 2015).

309 Soyasaponins are triterpenoid saponins commonly found in legume plants. They
310 are composed of aglycone and oligosaccharide moieties. Soyasaponins are subdivided
311 into four groups based on the aglycone structures: soyasaponin group A, group B, and
312 group E (derived from soyasapogenol A, soyasapogenol B, and soyasapogenol E,
313 respectively), and DDMP saponin consisting of soyasapogenol B with a DDMP (2,3-
314 dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) residue at the C-22 position of the
315 aglycone (Figure 2B). Soyasapogenol B is biosynthesized by the hydroxylation of β-
316 amyrin at C-22 and C-24, soyasapogenol A has an additional hydroxylation at C-21, and
317 soyasapogenol E has a carbonyl group at C-22 (Zhang and Popovich 2009; Seki et al.
318 2015; Krishnamurthy et al. 2019). These soyasapogenols are further diversified by
319 glycosylation catalyzed by UGTs to form the soyasaponin groups A, B, and E. DDMP
320 saponin is biosynthesized from group B saponin, possibly via a UGT encoded by the Sg-
321 9 locus (Sundaramoorthy et al. 2019) (Figure 4).

322 Cycloartenol synthase (CAS) catalyzes the cyclization of 2,3-oxidosqualene to
323 cycloartenol, which is a precursor for phytosterols including cholesterol, campesterol, and
324 β-sitosterol. Cholesterol is then oxidized and glycosylated to form steroidal saponins
325 (Figure 4). Steroidal glycoalkaloids, typically found in species of *Solanum* as toxic
326 substances, are also biosynthesized from cholesterol as a precursor but incorporate an

amine group at C-26 to generate aglycones such as tomatidine and solanidine (Harrison 1990; Cardenas et al. 2015), which are subsequently glycosylated at the C3 hydroxy group and accumulated as α-tomatine in tomato (*Solanum lycopersicum*) and α-solanine in potato (*Solanum tuberosum*) (Friedman 2006). Genes involved in saponin biosynthesis have been identified from various plant species (Thimmappa et al. 2014; Lee et al. 2019; Jozwiak et al. 2020; Chung et al. 2020) and are often organized as a cluster in the genome (Itkin et al. 2013; Nutzmann et al. 2018) (Akiyama et al. 2021).

Secretion into the rhizosphere

Transporters of saponins for vacuolar accumulation or secretion to the rhizosphere have not been identified so far (Francisco and Martinoia 2018). The transporter responsible for the relocation of α-tomatine from the vacuole to the cytosol has recently been identified in tomato (Kazachkova et al. 2021) (Figure 3). Secretion of soyasaponins from soybean roots and tomatine from tomato roots have been investigated both in hydroponic culture and in field conditions. The secretion of soyasaponins from soybean roots was discovered during a metabolomic analysis of soybean root exudates (Tsuno et al. 2018) as a strong peak on the total ion chromatogram that did not show clear UV absorption typical to aromatic rings. A detailed assessment of *m/z* values and MS/MS spectra, together with authentic standard specimens, identified this peak as soyasaponin Bb.

Soyasaponins are secreted during growth in hydroponic culture but peak at the early growth stage, as is the case for isoflavones of soybean (Tsuno et al. 2018) (Figure 4).. Soybean roots secrete both group A and group B soyasaponins, but the secretion of DDMP saponins is limited despite the predominant accumulation in soybean roots. The differential composition of soyasaponins between roots and root exudates suggests the

involvement of regulatory mechanisms such as transporters and apoplastic enzymes to be elucidated in future studies. Other legume plants including, but not limited to, *Lotus japonicus*, alfalfa (*Medicago sativa*), and pea (*Pisum sativum*), secrete soyasaponins at varying concentrations and compositions, but DDMP saponins are not detectable from root exudates in these legume species (Tsuno et al. 2018). The contents of the major soyasaponins, soyasaponin Ab and soyasaponin Bb, in root exudates have no apparent diurnal pattern, although the gene expression levels of β-amyrin synthase, cytochrome P450, and UDP-glucuronosyltransferase involved in soyasaponin biosynthesis (Krishnamurthy et al. 2019; Seki et al. 2015) are higher at night (Matsuda et al. 2020). The amount of soyasaponins in the rhizosphere of soybean grown in field is slightly increased during the growth stages, but the composition is stable, with group B soyasaponins representing about 60% of the total soyasaponins, followed by group A, E, and DDMP soyasaponins. Soyasaponin Bb represents up to 70% of group B soyasaponins, and soyasapogenols are limited throughout the growth stages (Fujimatsu et al. 2020).

Tomatine and its aglycone tomatidine were also found in root exudates of tomato (Kirwa et al. 2018). In hydroponically grown tomato, the concentrations of tomatine and tomatidine in root exudates are higher during the early growth stages than the later growth stages, consistent with the secretion of isoflavones and soyasaponins during growth (Nakayasu et al. 2021) (Figure 4). The regulations of tomatine secretion remain elusive, but it is known that it is regulated systemically by the addition of glycosylated azelaic and possibly by soil microbiota (Korenblum et al. 2020). In field-grown tomatoes, rhizosphere tomatine contents are comparable between the flowering and green-fruit stages. Neither tomatine nor tomatidine is detectable in bulk soil, suggesting that tomatine is secreted from field-grown tomato plants and accumulates in the rhizosphere throughout the growth

stages (Nakayasu et al. 2021).

Roles in the rhizosphere

Saponins exert a diverse range of biological properties relevant to rhizosphere interactions including antibacterial, antifungal, and insecticidal activities, in addition to various pharmaceutical effects in humans (Cheok et al. 2014; Vincken et al. 2007; Augustin et al. 2011). The ecological significance of saponins has long been of particular interest for their relevance in crop production. Biological activities against pathogens have been reported; for example, minutosides extracted from *Allium minutiflorum* have antimicrobial activities against various fungal and oomycete pathogens such as *Alternaria alternate*, *Botrytis cinerea*, *Fusarium oxysporum*, *F. solani*, *Pythium ultimum*, and *Rhizoctonia solani* (Barile et al. 2007), alliospirosides extracted from *Allium cepa* have antifungal activities against a range of fungi including *Botrytis cinerea* and *Colletotrichum gloeosporioides* (Teshima et al. 2013); and aescin from *Aesculus hippocastanum* shows antifungal activities against *Microdochium nivale*, *Pyrenophora teres*, and *Leptosphaeria maculans* (Trda et al. 2019). Additionally, steroidal glycoalkaloids such as α-solanine, α-chaconine, and α-tomatine show hatching stimulation activity in potato cyst nematode eggs, albeit weaker than solanoeclepin A, a triterpene secreted from potato roots (Shimizu et al. 2020).

Roles as allelochemicals

Saponins have also long been recognized as allelochemicals (Oleszek W. 1992). Saponins in the roots of alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) shows inhibitory effects on seed germination and seedling growth of wheat (*Triticum aestivum*)

399 (Oleszek and Jurzysta 1987; Oleszek 1993), and medicagenic acid saponins exhibit
400 autotoxic effects and significantly lower the germination rate of alfalfa seeds as well
401 (Ghimire et al. 2019). Ginsenosides from *Panax notoginseng* exhibit autotoxicity and
402 these saponins accumulated in soil are suggested to cause the replant failure of this plant
403 (Yang et al. 2015).
404
405 *Roles in modulating microbiota*
406 The function of saponins in modulating the rhizosphere microbiota has recently been
407 identified for saponins. An oat *sad1* mutant deficient in avenacin, a triterpenoid saponin,
408 harbors altered rhizosphere microbial communities, compared with the wildtype (Turner
409 et al. 2013). We used tomato as a model system to investigate the role of saponins in
410 shaping the rhizosphere microbiota. Tomatine and tomatidine treatments of field soil *in*
411 *vitro* resulted in the enrichment of ten and two families, respectively, and the depletion of
412 35 and 78 families, respectively. The bacterial communities of both tomatine- and
413 tomatidine-treated soils are more similar to those of the tomato rhizosphere than of bulk
414 soil, as shown by a smaller weighted UniFrac distance between PSM-treated soil and the
415 rhizosphere than between PSM-treated soils and bulk soil. Sphingomonadaceae was the
416 only family enriched in both PSM-treated soil and tomato rhizosphere soil. Among
417 Sphingomonadaceae, the genus *Sphingobium* was particularly enriched, and this increase
418 was attributable to one amplicon sequence variant (ASV) among the 15 ASVs observed
419 in these soil samples (Figure 4). Sphingomonadaceae was also enriched in soil treated
420 with soyasaponin Bb (Fujimatsu et al. 2020); operational taxonomic units annotated as
421 *Novosphingobium* were particularly enriched in soyasaponin Bb-treated soil as well as
422 the soybean rhizosphere (Figure 4). It is of particular interest to investigate whether

differences in the carbon skeletons of saponins affect the influence of saponins in rhizosphere bacterial communities. While the effect on bacterial communities is not known, ginsenosides (triterpenoid saponins in Sanqi ginseng) alter fungal communities with the enrichment of potentially pathogenic taxa including *Alternaria* and *Fusarium*, and with the depletion of potentially beneficial taxa such as *Acremonium*, *Mucor*, and *Ochroconis* (Li et al. 2020), which is a potential cause of replanting failure in Sanqi ginseng.

Dynamics of flavonoids and saponins in the rhizosphere

The dynamics and interactions of PSMs and microbes in the rhizosphere are of particular importance for gaining insight into the functions of PSMs. Traditionally, the amount of PSMs secreted from roots has been analyzed using hydroponic cultures because of the ease of applicability compared with field sampling (Oburger and Jones 2018). Although research in our laboratory has revealed that the secretion of daidzein from roots follows a similar pattern during the growth stages (i.e., higher secretion in early growth stages than later growth stages), the amount of daidzein secreted from roots was much higher in the field (Toyofuku et al. 2021), pointing toward the importance of measurement of metabolites in field-grown plants. Along with the differences in the amount and rate of secretion, it is technically challenging to analyze the positional secretion rate using a hydroponic system. In hydroponic culture, the total amount of metabolites secreted from whole roots is usually measured; in reality, the secretion rate varies based on root area (Pearson and Parkinson 1960; McDougall and Rovira 1970; Weisskopf et al. 2005). Recently, analysis using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) showed a distribution of various metabolites including PSMs in the

447 rhizosphere (Velickovic et al. 2020). In this study, a polyvinylidene fluoride (PVDF)
448 membrane was placed against the soil-root interface of plants grown in a rhizobox. The
449 PVDF membrane was then mounted on a MALDI target plate for mass imaging.
450 Biosensors are also useful for revealing the distribution of metabolites in the rhizosphere.
451 Fux fusion bioreceptors have been developed in *Rhizobium leguminosarum* to detect
452 specifically for sugars, polyols, amino acids, organic acids, and flavonoids (Pini et al.
453 2017). Although a bacterial association with the plant root system needs to be validated,
454 these biosensors are potentially applicable for a broader range of bacteria and metabolites
455 to investigate the spatiotemporal distribution of PSMs in the rhizosphere.
456
457 *Degradation*
458 The amount of secretion and the stability of PSMs need to be analyzed to understand the
459 dynamics of PSMs in the rhizosphere. Once secreted, PSMs are degraded by soil microbes.
460 Despite the rather toxic properties of flavonoids and saponins, these PSMs are also a
461 carbon source for microbes possessing catabolic enzymes. The stability of PSMs in the
462 rhizosphere varies depending on the metabolites and soil microbial communities. In
463 contrast to primary metabolites that are degraded within a few minutes (Gunina and
464 Kuzyakov 2015), flavonoids are rather stable in the soil. The majority of naringenin and
465 formononetin is degraded within 96 hours (Shaw and Hooker 2008), while the half-life
466 of apigenin, kaempferol, and their derivatives ranges from 4.4 to 14.7 days with a seasonal
467 variation (Sosa et al. 2010). The half-life of daidzein is about seven days, measured in
468 soil collected from a soybean farm (Okutani et al. 2020) (Figure 3). The half-life of
469 saponins in the soil is comparable with flavonoids. The allelopathic effects of saponins
470 from alfalfa and red clover last for one to two weeks depending on the soil (Oleszek and

Jurzysta 1987), and most soyasaponin Bb and tomatine in soil are degraded within a week (Fujimatsu et al. 2020) (Nakayasu et al. 2021).

Aerobic flavonoid biodegradation has been reported for a wide range of bacterial species (Shaw et al. 2006). *Nod* gene-inducing daidzein and genistein are also to be degraded by rhizobial species via multiple C-ring fission, although genes involved in this pathway have not been identified (Rao and Cooper 1994, 1995). The metabolic pathways for flavonoid degradation have been characterized in intestinal microbes; daidzein, when consumed as a part of soy products, is converted to a reduction product, equol, and a C-ring cleavage product, *O*-demethylangolensin, by anaerobic intestinal bacteria (Feng et al. 2018). These products have not been identified in rhizosphere soil to our knowledge. Screening of mutants defective in naringenin catabolism in *Herbaspirillum seropedicae* revealed the involvement of a monooxygenase, FdeE, in the cleavage of naringenin, possibly together with FdeD, a putative Rieske protein in fed operon (Marin et al. 2013; Marin et al. 2016). Other proteins encoded by the fde operon include FdeH, a cupin family protein that contains quercetin 2,3-dioxygenase for cleaving quercetin into 2-protocatechuoyl-phloroglucinol carboxylic acid and carbon monoxide, identified in *Bacillus subtilis* (Bowater et al. 2004; Barney et al. 2004) and *Streptomyces* sp. (Merkens et al. 2007). Most of the genes involved in flavonoid catabolism, especially those for isoflavones, have not been identified.

Intestinal and fecal microorganisms capable of metabolizing saponins have also been reported (Hu et al. 2004; Dong et al. 2017; del Hierro et al. 2018). Sapogenins such as oleanolic acid, hederagenin, serjanic acid, diosgenin, and soyasapogenol B accumulate when plant extracts rich in saponins (e.g., quinoa, lentil, and fenugreek) are fermented by gut microbiota (del Hierro et al. 2020). Sapogenins are absorbed by the gastrointestinal

tract, and no gut microorganisms capable of degrading sapogenin have been identified. In contrast, it is not well understood what kind of microorganisms decompose compounds in the soil. Microorganisms capable of degrading tomatine into tomatidine have been reported (Ford et al. 1977; Okmen et al. 2013), and *Sphingobium* spp. isolated from tomatine-treated soil degrade tomatine and tomatidine and use them as a carbon source (Nakayasu et al. 2021). Saponins such as ginsenosides and soyasaponins, are also metabolized by intestinal and fecal microbes (Hu et al. 2004; Dong et al. 2017). Yet, the pathways and genes involved in the degradation of aglycones have not been characterized, particularly for rhizosphere microbes.

Adsorption

In addition to the degradation by soil microbes, adsorption by organic matter and clay minerals reduces the distribution of metabolites in the rhizosphere. We used daidzein as a model to investigate the adsorption to grey lowland soil collected from a soybean farm. Possible adsorption sites are humic substances and clay minerals. Humic substances contain hydroxyl and phenolic hydroxyl groups involved in the formation of complexes with organic substances (Pei Gan and Yau Li 2013). Decomposition of organic matter in grey lowland soil reduced daidzein adsorption, suggesting the involvement of humic substances in limiting the distribution of daidzein in the rhizosphere. In contrast, adsorption of daidzein to clay minerals such as kaolinite, a 1:1-type silicate mineral, is much lower than that to grey lowland soil, and adsorption to illite, a 2:1-type silicate material, is undetectable (Okutani et al. 2020) (Figure 3).

Simulation

519 The distribution of mineral ions and water in the soil surrounding plant roots has been
520 simulated (Duncan et al. 2018; Zarebanadkouki et al. 2014; Vereecken et al. 2016). For
521 PSMs in rhizosphere soil, the advection-diffusion (dispersion) equation has recently been
522 used to simulate the dynamics of daidzein in the rhizosphere (Okutani et al. 2020). A
523 single root of diameter 2 mm and length 10 cm was set in the center of soil with a diameter
524 of 20 cm and a depth of 20 cm. The root length and diameter were assumed to be constant,
525 and daidzein secretion was assumed to be equal from all parts of the roots. Daidzein
526 distribution was predicted to within a few millimeters of the root surface during the early
527 growth stages (14 days). Although daidzein distribution was shown to be within 2 mm in
528 a rhizobox experiment (Okutani et al. 2020), this simulation is based on constant soil
529 environmental conditions, the absence of root growth, and equal secretion from all parts
530 of the roots. It is necessary to incorporate, at a minimum, the soil water contents (i.e., wet
531 conditions on rainy days and dry conditions on sunny days) and root growth with differing
532 secretion rates of metabolites to precisely simulate the distribution of metabolites in
533 rhizosphere soil in field-grown conditions. Also, simulation based on the secreted amount
534 of daidzein in hydroponic culture underestimates the amount of rhizosphere daidzein.
535 When the daidzein secretion rate measured in field-grown soybean was used for the
536 simulation, the estimated daidzein concentration in the soybean rhizosphere was within
537 the range of that from the rhizosphere in the soybean field (Toyofuku et al. 2021). So far,
538 the adsorption coefficient has been analyzed for flavonoids and saponins in addition to
539 organic xenobiotics and estrogens in soil (Shaw and Hooker 2008; Caron et al. 2010)
540 (Fujimatsu et al. 2020); thus, simulations based on a fluid model can be applied to predict
541 the rhizosphere distribution of PSMs.
542

Conclusion and future perspectives

Research in the past few decades has identified a vast array of roles of flavonoids and saponins in the rhizosphere, especially for mediating interactions between plants and microbes. Recent advancement of multi-omics analysis revealed a tight network between host plants and microbiota, which opens up a holistic approach toward a comprehensive understanding of plants and microbes. This concept considers the host plant and its microbiota as a unique biological entity called "holobiont," in which the host and microbiota interact to affect morphology, development, and physiology among others (Rosenberg and Zilber-Rosenberg 2016; Hassani et al. 2018). It remains largely unclear how the metabolic network in the holobiont is established and how it affects plant growth, fitness, and robustness to changing environments. For crop species, the domestication process has affected root microbiota, mediated at least partially by the alteration of root exudates (Iannucci et al. 2017; Escudero-Martinez and Bulgarelli 2019). The metabolic network in the holobiont provides a valuable basis for designing an optimized microbiota to confer robustness against both biotic and abiotic stresses and, eventually, to improve crop yields. Flavonoids and saponins are key metabolites for such an approach since these bioactive compounds mediate the interaction between the plant and the microbiota, and recent evidence has revealed the possibility of fine-tuning the interactions (Fujimatsu et al. 2020; Nakayasu et al. 2021).

The direct application of metabolites is the first step toward this goal. Flavonoids applied to seeds improve the nodulation in several legume crops (Mabood et al. 2014), and saponins applied to seeds improve salinity stress tolerance in quinoa and soybean (Yang et al. 2018; Soliman et al. 2020). There remain possibilities to utilize these PSMs in agriculture directly, but an obstacle is the stability of these metabolites in the soil.

567 Multiple applications are necessary to exert bioactive properties during a crop season, and
568 the application of PSMs to the rhizosphere is technically unfeasible. Designing a
569 holobiont-metabolic network utilizing both plant breeding and microbial inoculation
570 would be promising to circumvent these problems. Designing rhizosphere microbiomes
571 for crop productions has been proposed in reviews (Mueller and Sachs 2015; de Souza et
572 al. 2020; Pascale et al. 2020), and the holobiont-metabolic network could be a key
573 component.

574 Metagenomic analysis revealed key bacterial taxa in the microbiome for
575 beneficial traits. By comparing the rhizosphere metagenomes of resistant and susceptible
576 tomato varieties to pathogenic *Ralstonia solanacearum,* it was found that *Flavobacterium*
577 was abundant in the rhizosphere of resistant varieties, and isolated *Flavobacterium*
578 suppressed *R. solanacearum* when inoculated in pots (Kwak et al. 2018). Metagenomics
579 on disease suppressive soil identified a consortium of *Chitinophaga* and *Flavobacterium*
580 for the suppression of the fungal root pathogen *Rhizoctonia solani*, and gene clusters
581 encoding the production of nonribosomal peptide synthetases and polyketide synthases
582 in *Flavobacterium* were found to be essential for disease suppression (Carrion et al. 2019).
583 In contrast to metagenomics, the identities and functions of metabolites in the rhizosphere
584 largely remained unknown because of the instability and limited amounts of metabolites
585 in soil and difficulties with extraction from the rhizosphere. In addition to PSMs,
586 microbial metabolites exert diverse ranges of influence on host plants and co-occurring
587 microbes (Backer et al. 2018; Weisskopf et al.). The rhizosphere metabolome is a
588 prominent approach for uncovering the novel metabolites in the rhizosphere. We recently
589 found okaramine A, B, and C in the rhizosphere of hairy vetch (*Vicia villosa*), a cover
590 crop or a green manure crop (Sakurai et al. 2020). Okaramines were first identified as

591 insecticides from *okara* inoculated with *P. simplicissimum* AK-40 (Hayashi et al. 1989),
592 but they had not been identified in nature. Okaramine B was also detected in the
593 rhizosphere of soybean grown after hairy vetch but not in soybean without previous hairy
594 vetch cultivation, suggesting an interspecies soilborne legacy or an indirect defense of
595 plants against pests (Sakurai et al. 2020; Matsuda 2018).

596 Multi-omics analyses integrating metagenomics and rhizosphere metabolomics
597 are prerequisites for designing microbiomes based on holobiont-metabolic networks. The
598 rhizosphere is vital for plant growth and crop production. Unlike the accumulation of
599 microbial genomics, metabolomics has only revealed the tip of the iceberg. It will be of
600 particular importance to design a holobiont metabolic network by combining the
601 identification of new key metabolites, metagenomic analysis, isolation, and functional
602 characterization, and plant breeding to enable plants to grow more robustly (Figure 5).
603

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617

618 **Disclosure statement**

619 No potential conflict of interest was reported by the author.

620

621 **Data Availability Statement**

622 The data generated during this study are available from the corresponding author upon
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624

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Graphical Abstract Caption

Flavonoids and saponins are secreted into rhizospheres. These plant specialized metabolites exert various functions in mediating interactions with microbiota.

Figure legends

Figure 1. Soybean rhizosphere. Isoflavones and soyasaponins are secreted into the rhizosphere (①) and affect the bacterial communities (②).

Figure 2. Chemical structures of isoflavones in soybean

Figure 3. Secretion and fate of daidzein in soybean rhizosphere. (1) Sugiyama et al. (2007), (2) Suzuki et al. (2006), (3) Sugiyama et al. (2006), (4)Toyofuku et al. (2021), (5) Matsuda et al. (2020), (6) Sugiyama et al. (2017), (7) Okutani et al. (2020).

Figure 4. Secretion of soyasaponin from soybean roots and tomatine from tomato roots: cycloartenol synthase (CAS), β-amyrin synthase (BAS), UDP-sugar dependent glycosyltransferase (UGT), arabinose (Ara), galactose (Gal), glucose (Glc), glucuronic acid (GlcA), rhamnose (Rha), xylose (Xyl). CAS and BAS are members of OSC family.

1068 Bold arrows represent multiple reactions.

1069

1070 Figure 5. Integration of multi-omics to design holobiont metabolic networks that promote

1071 plant growth and suppress pathogens.

1072