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1 **The coumarins: secondary metabolites playing a primary role in plant nutrition and**
2 **health**

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15 **KEY WORDS:** *Arabidopsis thaliana*, coumarins, iron, PDR9

16

17 **ABSTRACT**

18 Although abundant in soils, iron (Fe) is poorly bioavailable for plants. Improving Fe uptake in
19 crops, enabling them to grow in Fe-depleted soils, has become a major focal interest. The
20 secretion of Fe-mobilizing coumarins by plant roots recently emerged as an important factor
21 allowing non-grass species to cope with low Fe bioavailability. The main molecular actors
22 involved in the biosynthesis and secretion of coumarins have been identified but the precise
23 regulatory mechanisms that tune their production remain poorly understood. Herein are
24 reviewed the recent progress on coumarins synthesis and transport in plants and future
25 research directions to gain knowledge on these mechanisms, which will offer novel
26 opportunities for improving plant growth and health and for generating Fe-fortified crops.

27

28 **Iron acquisition**

29 Iron (Fe) is an essential micronutrient for plant productivity as well as for the quality of their
30 derived products [1]. This is because Fe is necessary for the activity of several
31 metalloproteins that participate in numerous fundamental biological processes such as
32 photosynthesis, mitochondrial respiration, DNA repair and replication or nitrogen and sulfur
33 assimilation [2]. Although Fe abundance is high in most soils (Fe is the fourth most abundant
34 element in earth crust), Fe availability remains low for plants because it is mainly present in

35 the form of insoluble Fe hydroxides and oxides [3]. This is for instance the case in well-
36 aerated **alkaline** or **calcareous** soils that represent one-third of the world's cultivated lands.
37 As a consequence, plants growing on these soils display growth and developmental defects
38 together with reduced Fe concentration. Considering that about one billion people worldwide
39 suffer from Fe-deficiency anemia, a reduction of Fe concentration in staple crops is a major
40 threat for several populations, in particular those whose dietary intake of Fe relies mainly or
41 entirely on plants. Therefore, a better understanding of the mechanisms evolved by plants to
42 acquire Fe is needed to improve Fe nutrition in crops, the entry point of Fe in human diet.

43

44 Fe acquisition is the first limiting step for Fe nutrition in plants. Monocots and dicots have
45 evolved different strategies to ensure this first step. For instance, non-grass species have
46 evolved a reduction-based mechanism to solubilize and absorb Fe from the soil [4, 5]. This
47 mechanism relies on the release of protons into the **rhizosphere** to acidify the root
48 surrounding media and increase the concentration of Fe(III) in the soil solution. The
49 solubilized Fe^{3+} is then reduced into Fe^{2+} at the root surface prior to its transportation across
50 the root epidermal cell membrane. In arabidopsis (*Arabidopsis thaliana*), these three steps are
51 tightly connected and respectively catalyzed by the AHA2 proton-ATPase, the FERRIC
52 REDUCTION OXIDASE 2 (FRO2) and the IRON-REGULATED TRANSPORTER 1
53 (IRT1) [6] (Figure 1).

54

55 In non-grass species, the secretion of **secondary metabolites** with Fe-mobilizing properties
56 appeared to play a wider role than what was previously thought [7]. A good example are the
57 coumarins, which have emerged as key players for Fe solubilization and/or uptake, in
58 particular when the pH of the soil is high and the FRO2 activity is reduced. Coumarins
59 secretion in arabidopsis requires the PLEIOTROPIC DRUG RESISTANCE 9/ATP-
60 BINDING CASSETTE G37 (PDR9/ABCG37) transporter [7-9]. Herein, are reviewed recent
61 findings, mainly obtained in Arabidopsis, ranging from the biosynthesis, storage and
62 trafficking of the different coumarins involved in plant Fe acquisition to the mechanisms that
63 regulate their biosynthesis and secretion.

64

65 **The coumarins biosynthetic pathway**

66 Several functions necessary for the plant responses to various biotic and abiotic stresses as
67 well as for the plant structural support or the regulation of its growth are ensured by
68 metabolites originated from the **phenylpropanoid** pathway. The phenylpropanoid

69 biosynthetic pathway is relatively well described for several classes of molecules (e.g. lignins,
70 flavonoids); however, the steps leading to the biosynthesis of coumarins were, until recently,
71 poorly documented. Expression studies highlighted that several genes from the
72 phenylpropanoid pathway are responsive to Fe availability [10, 11], in particular those from
73 the main core of the pathway that leads to the biosynthesis of 4-coumaroyl-CoA (e.g. PAL1:
74 PHENYLALANINE AMMONIA-LYASE 1, 4CL1 and 2: 4-COUMARATE:CoA LIGASE 1
75 and 2), the first intermediate in common between the different classes of phenylpropanoid
76 compounds (Figure 2). The HYDROXYCINNAMOYL-CoA SHIKIMATE/QUINATE
77 HYDROXYCINNAMOYLTRANSFERASE (HCT) and the CAFFEYOYL-CoA O-
78 METHYLTRANSFERASE (CCoAOMT1) are responsive to Fe regime and ensure the
79 conversion of 4-coumaroyl-CoA into feruloyl-CoA, the precursor of coumarins. For instance,
80 the accumulation of coumarins in *ccoamt1* loss-of-function mutants is impaired [12, 13].

81
82 The FERULOYL-CoA 6'-HYDROXYLASE 1 (F6'H1) catalyzes the first step committed to
83 the biosynthesis of coumarins (Figure 3), leading to the formation of 6'-hydroxyferuloyl-CoA
84 [13]. The characterization of *f6'h1* loss-of-function mutants has allowed demonstrating that
85 coumarins play an important role in plant Fe nutrition, in particular in response to Fe shortage
86 in alkaline soils [14, 15]. In Arabidopsis, three coumarins with Fe-mobilizing properties are
87 synthesized. These molecules are the fraxetin (7,8-dihydroxy-6-methoxycoumarin), the
88 sideretin (5,7,8-trihydroxy-6-methoxycoumarin) and the esculetin (6,7-dihydroxycoumarin)
89 [16-18]. Each one of these molecules displays a catechol moiety (i.e. two neighboring
90 hydroxyl groups) that interacts with and mobilizes the Fe present in soils. Until recently, the
91 biochemical steps leading to the biosynthesis of these catechol coumarins were still to be
92 characterized. Recent studies have shed light on this part of the coumarin pathway
93 highlighting that scopoletin, a non-catechol coumarin, is the precursor of fraxetin and
94 sideretin. Scopoletin is issued from the trans-cis isomerization and lactonization of 6'-
95 hydroxyferuloyl-CoA. This step is partially spontaneous but the reaction efficiency is
96 significantly increased by the activity of COUMARIN SYNTHASE (COSY), a BHAD
97 acyltransferase enzyme [19]. The hydroxylation at the C8 position of scopoletin by the
98 SCOPOLETIN 8-HYDROXYLASE (S8H), a 2-oxoglutarate-dependent dioxygenase, leads to
99 the formation of fraxetin [17]. While alkaline pH favors the biosynthesis of fraxetin, the main
100 catechol coumarin secreted at acidic pH is sideretin. Sideretin biosynthesis involves the
101 activity of CYP82C4 [16, 20], an Fe deficiency-responsive cytochrome P450 enzyme whose
102 expression is abolished when plants are grown on high pH media [21]. How esculetin

103 biosynthesis occurs is still a matter of debate. It was demonstrated that COSY could catalyze
104 in vitro the spontaneous conversion of 6'-hydroxycaffeoyl-CoA into esculetin, but this step
105 has not yet been proven to occur in plants [19, 22]. In addition, it cannot be excluded that
106 esculetin derives from scopoletin following a demethylation step or from a side activity of
107 F6'H1 using caffeoyl-CoA as precursor [16].

108

109 **Coumarins storage and secretion**

110 Once synthesized, coumarins are either stored within the plant cells as glycosylated
111 conjugates (e.g. scopolin, fraxin, sideretin glycosides or esculin) to avoid toxicity or secreted
112 into the rhizosphere as **aglycones** (e.g. scopoletin, fraxetin, sideretin or esculetin) [23].

113

114 Within the plant cells, coumarins accumulate in the vacuolar compartment [24-27]. In
115 Arabidopsis, BGLU42 (BETA GLUCOSIDASE 42), which is predominantly expressed in
116 **trichoblasts** (root hair cells), is responsible for the deglycosylation of scopolin [28, 29].
117 *bglu42* loss-of-function mutants are unable to secrete scopoletin and accumulate large
118 amounts of scopolin [28]. Three additional beta glucosidases that are expressed in roots (i.e.
119 BGLU21, BGLU2122 and BGLU23) display a similar activity in vitro to that of BGLU42
120 toward scopolin and esculin [30]. Nevertheless, in vivo validations are still missing to
121 conclude on the biological role of BGLU21, BGLU22 and BGLU23, leaving unidentified the
122 enzymes involved in the deglycosylation of fraxin, sideretin glycosides or esculin prior to
123 their secretion into the rhizosphere. Coumarins secretion occurs mainly via the PDR9
124 transporter [31, 32]. Whether some specificity towards the secretion of the different
125 coumarins occurs between trichoblast (root hairs) and atrichoblast (non-root hairs) cells was
126 an open question until recently. By coupling spectral imaging (allowing monitoring iron-
127 mobilizing coumarins and scopolin accumulation in roots) with coumarins quantification in
128 root hair mutants, it was demonstrated that root hairs play a major role in scopoletin secretion
129 whereas fraxetin and esculetin exudation occurs through all epidermis cells [27].

130

131 Finally, it is noteworthy that small amounts of glycosylated coumarins have been identified in
132 the media surrounding the plant root system, in particular in response to Fe deficiency [28, 33,
133 34]. These observations suggest that a fraction of the glycosylated coumarins could be
134 directly secreted across the root epidermis (via an unknown mechanism) or that coumarin
135 aglycones could be glycosylated outside the plant roots [28, 34]. The presence of coumarins

136 in the growth medium of the *pdr9-2* mutant also indicates that others transporters might be
137 involved in coumarins secretion [31, 34].

138

139 **Coumarins cellular localization and trafficking**

140 Coumarins are essentially synthesized in roots, a tissue that coordinates Fe uptake with the
141 demand of the plant and that is in contact and influenced by soil microorganisms.
142 Underground, coumarins have emerged as important players for both optimizing Fe uptake
143 and shaping the root microbiome, thus affecting plant health [14, 15, 35]. In aerial tissues,
144 coumarins are involved in the plant response to pathogens [12, 35]. Their various roles in
145 different tissues suggested that these compounds might be highly mobile. However, very little
146 was known until recently on the actual localization and the trafficking of coumarins within the
147 plant body.

148

149 The precise pattern of scopolin, fraxin and esculin accumulation in roots was recently
150 uncovered [27]. These observations, in the light of F6'H1 localization studies [15], indicate
151 that scopolin, fraxin and esculin biosynthesis and storage preferentially occur in the cortex
152 cells, suggesting that the epidermis is mostly a transit tissue before their secretion into the
153 rhizosphere [27] (Figure 4). Whether this is also the case for the biosynthesis and exudation of
154 sideretin remains to be determined. However, there is an apparent discrepancy between the
155 proposed model and the expression of *S8H* and *CYP82C4*, which is mainly restricted to the
156 root epidermis [16]. Since it cannot be excluded that the pattern of *S8H* and *CYP82C4*
157 accumulation might differ from gene expression, protein localization studies will be necessary
158 in order to fully conclude. An in depth analysis of *COSY* protein accumulation would also be
159 useful since, to date, *COSY* promoter activity in plant grown under Fe replete condition is
160 only available, and show a ubiquitous expression within the roots (i.e. expressed in all cell
161 types in the differentiation zone) [19].

162

163 Coumarins accumulation was also observed in the endodermis in plants grown under replete
164 or Fe deficiency conditions, indicating that coumarins move also from the cortex to this cell
165 layer [27]. Cold and osmotic stresses as well as high carbon availability also stimulate
166 scopolin accumulation in roots, mainly in the endodermis [12], suggesting that scopoletin, or
167 its precursors (i.e. 6'-hydroxyferuloyl-CoA), might move from the cortex to the endodermis
168 via an Fe-independent mechanism.

169

170 Reciprocal grafting experiments between wild type and *f6'h1* mutant seedlings allowed
171 demonstrating that coumarins movement also occurs between different organs of the plant,
172 from roots to shoots (Figure 4) [27]. The identification of both scopolin and scopoletin in
173 xylem sap further confirmed this assertion [27]. Interestingly, these reciprocal grafting
174 experiments highlighted also that scopolin can be synthesized in leaves via a mechanism that
175 is independent of F6'H1 activity in roots. These findings therefore suggest that the
176 accumulation of scopolin in leaves in response to biotic (e.g. pathogens) and abiotic (e.g.
177 cold) stresses [12, 35] might also derive, at least in part, from the roots. However, it is
178 noteworthy that since *F6'H1* expression is induced in leaves following **2,4-D** treatment [13],
179 it might be that scopoletin is synthesized in this tissue in a F6'H1-dependent manner in some
180 specific growth conditions. When exogenously applied at the surface of arabidopsis
181 cotyledons, fraxin and esculin move to the roots *via* the phloem, suggesting that coumarins
182 could relocate from the aerial tissues to the roots [25, 36]. Whether this observation is
183 relevant or not for plants is still a matter of debate.

184

185 Taken together, these different studies highlight that coumarins accumulation and trafficking
186 is a complex and dynamic process. Beside these recent findings, our understanding of
187 coumarins trafficking in plants remains a jigsaw for which several pieces are missing (Figure
188 1 and 4). For instance, the transporters and the glycosyltransferases that participate to the
189 compartmentalization of the different coumarins into the vacuoles are still to be characterized.
190 Additional glucosidases, other than BGLU42, are likely to be involved in the secretion of
191 fraxetin, sideretin and esculetin into the root surrounding media. In addition, if it has been
192 demonstrated that the role of PDR9 is not restricted to the secretion of coumarins into the
193 rhizosphere, but also extent to their transportation from the cortical cells to the epidermis [27],
194 the molecular mechanisms by which coumarins move from the cortex cells to the endodermis,
195 and then to the xylem sap and the aerial tissues, is still to be elucidated.

196

197 Recent studies also support that secreted coumarins are uptaken by surrounding plants. This
198 was for instance demonstrated for the umbelliferone (7-hydroxycoumarin), a non-catechol
199 coumarin [37]. Since the addition of scopoletin into the growth media is sufficient to rescue
200 the chlorotic phenotype of *f6'h1-1* but not the one of *s8h* [16, 18], it was hypothesized that
201 scopoletin is taken up by the root of the *f6'h1-1* mutant and then metabolized into catechol
202 coumarins such as fraxetin and/or sideretin to facilitate Fe acquisition. The ability of the
203 *f6'h1-1* mutant to uptake, glycosylate and store scopoletin, fraxetin and esculetin in roots was

204 recently demonstrated [27]. Interestingly, in this study it was also shown that other dicot (i.e.
205 tomato) and gymnosperm (i.e. maritime pine) species had, in contrast to monocots, the ability
206 to uptake the main Fe-mobilizing coumarin found in Arabidopsis, fraxetin. These later results
207 indicate that the ability of plants to uptake coumarins has most probably emerged early during
208 the evolution of land plants. It also suggests that this ability has been lost in graminaceous
209 plant species. Whether coumarins uptake requires a dedicated transport system or occurs by
210 simple diffusion will have to be determined.

211

212 **The potential roles of coumarins in Fe acquisition**

213 Although the importance of catechol coumarins secretion for Fe acquisition has been
214 demonstrated in several studies [16, 18, 31], their precise function in Fe nutrition is not fully
215 understood.

216

217 The ability of catechol coumarins (i.e. sideretin, fraxetin, esculetin) to reduce Fe^{3+} has clearly
218 been established [16]. However, recent advances indicate that reducing Fe might not be the
219 sole role for the secreted coumarins in plant Fe nutrition. For instance, no difference in the
220 ferric reductase activity of wild type and *f6'h1-1* mutant was observed and the use of secreted
221 phenolics from wild type plant subjected to Fe deficiency was inefficient to rescue *fro2*
222 mutant chlorosis [15, 32]. Importantly, catechol coumarins can form complexes with Fe(III)
223 at alkaline pH (mostly in a 2 coumarins to 1 Fe stoichiometry, [38]), thus facilitating Fe
224 dissolution [16], which raises an important question: How can this mechanism improve Fe
225 nutrition for plants, considering that alkaline pH reduces *FRO2* expression [21] and
226 inactivates the activity of the encoded enzyme? [8, 9]

227

228 An appealing hypothesis would be that at alkaline pH, Fe-catechol coumarin complexes are
229 transported into the plant roots through an unknown mechanism. Since, as aforementioned,
230 some direct evidences support that catechol coumarins can be uptaken by plant roots [27] and
231 since co-cultivation of wild type plants with *f6'h1* is sufficient to revert the chlorotic
232 phenotype of the mutant [14, 19], it might be that the role of coumarins secretion at alkaline
233 pH, in addition to Fe dissolution and reduction, could be the chelation and transport of Fe into
234 the plant roots. Indeed, this hypothesis has not yet been demonstrated and additional
235 experiments will be required in order to fully conclude.

236

237 **Impact of other mineral nutrients on coumarins biosynthesis**

238 Plants must sense and adapt their responses to changes in external and internal Fe
239 concentrations to adjust their growth and match resources present in the soil. The plant
240 responses to fluctuations in Fe accessibility are therefore tightly dependent on the availability
241 of other macro- and micronutrients [39-41]. The interdependency between mineral nutrients
242 raises questions on whether the levels and the identity of coumarin compounds produced by
243 plants and secreted into the rhizosphere is modulated under single and combined nutrient
244 stress conditions.

245

246 For instance, an excess of zinc (Zn) in the growth media of arabidopsis seedlings is sufficient
247 to promote coumarins synthesis and excretion [42]. This effect is accompanied by an increase
248 of *FRO2* and *IRT1* expression and ferric chelate reductase activity, indicating that Zn excess
249 induces the Fe deficiency responses. Similar but less marked responses were observed with
250 plants treated with excess of nickel (Ni) and cobalt (Co) and, to a lesser extent, with cadmium
251 (Cd). On the contrary, manganese (Mn) deficiency provokes a decrease of S8H protein
252 accumulation [43], which suggests that Mn deficient plants may produce less fraxetin and
253 sideretin than plants grown in Mn sufficient condition.

254

255 The availability of different nutrients can simultaneously vary resulting from the very low
256 concentration of two or more nutrients in the soil and/or the competition that exists between
257 plants and living organisms for mineral nutrients, making them less available to plants [44].
258 For instance, arabidopsis plants grown on medium lacking P or Fe showed shorter primary
259 root length phenotype compared to plants grown in presence of P and Fe (control condition)
260 [39], while plants growing under combined Fe and P deficiencies (both elements were
261 removed from the medium) displayed a remarkable recovery of the primary root elongation
262 [45]. This indicates that combined nutrient stress is a new condition, and not the simple
263 addition of the two stresses. Notably, the integration of nutrient stresses can be observed at
264 the metabolic level as well. Early work showed that in addition to Fe deficiency, arabidopsis
265 plants subjected to P deficiency exudate coumarins [46]. Nevertheless, differences between
266 the types and the levels of coumarins produced and secreted in response to Fe, P or Fe and P
267 deficiencies were observed [46, 47]. For instance, considering the main catechol coumarins,
268 both fraxin and sideretin glucosides accumulate in roots at high levels in response to Fe
269 deficiency whereas under P deficiency, fraxin level remains stable, or slightly reduced
270 compared to control condition, and sideretin glucosides accumulation decreases. Under Fe
271 and P dual deficiency, fraxin level is similar to that of Fe deficiency but sideretin glucosides

272 accumulation is lowered [46, 47]. Scopolin accumulation is solely affected by P availability,
273 whether it is alone or combined. A role of Fe-deficiency response regulators in the regulation
274 of Fe and of P deficiency-induced coumarin profiles has been observed, and similar
275 observation has been made with the P deficiency response regulators [47]. From these data, it
276 is hypothesized that an antagonistic cross talk between Fe and P deficiency responses
277 modulates the biosynthesis of coumarins [47], in particular the catechol ones, which is in
278 adequacy with reported effects of P concentration in the growth media on Fe availability (i.e.
279 under P deficiency Fe availability increases) [48]. However, such hypothesis does not apply
280 when plants are subjected to P and Fe dual deficiency, suggesting that P availability interferes
281 with Fe deficiency-induced biosynthesis of catechol coumarins [47].

282

283 **Transcriptional regulation of coumarins biosynthesis**

284 Even though the induction of coumarins biosynthesis occurs essentially at the transcriptional
285 level [15, 18, 49], little is known on the actual molecular actors that are involved.

286

287 In arabidopsis, several bHLH transcription factors regulate the Fe deficiency responses in a
288 complex regulatory network [49-52] (Figure 5). Among the main regulators of these
289 responses are FIT/bHLH29 (FER-LIKE IRON DEFICIENCY-INDUCED
290 TRANSCRIPTION FACTOR) and the newly described URI/bHLH121 (UPSTREAM
291 REGULATOR OF IRT1), two transcription factors whose activity relies on their
292 phosphorylation state [51, 53, 54]. URI is necessary to activate the expression of several
293 regulatory genes involved in the control of Fe homeostasis, indirectly modulating the
294 expression of genes involved in Fe uptake (e.g. *FRO2*, *IRT1*), transport (e.g. *NAS4*:
295 *NICOTIANAMINE SYNTHASE 4*) and storage (e.g. *FER1*: *FERRITIN 1*) as well as in the
296 biosynthesis (i.e. *F6'H1*, *S8H* and *CYP82C4*) and secretion (i.e. *BGLU42*) of coumarins [49].
297 Following Fe starvation, the induction of FIT expression relies, at least in part, on the activity
298 of URI [49]. FIT activity is necessary to activate the expression of *S8H* and *CYP82C4* [15, 49,
299 55]. As a consequence, *fit* and *uri* mutants display very low levels of the two main catechol
300 coumarins, fraxin and sideretin glycosides [15, 49]. However, since *F6'H1* expression is only
301 partially controlled by FIT, *fit* mutants still accumulate scopolin and esculin [47]. In contrast,
302 *F6'H1* expression is tightly regulated by the MYB72 transcription factor and *myb72* mutant
303 produces almost no scopolin [28]. It is also likely that the expression of key genes involved in
304 esculin biosynthesis is under the control of MYB72 since esculin accumulation is
305 completely abolished in *myb72* mutant [28]. However, it cannot be excluded that the absence

306 of esculetin in *myb72* mutant roots might be a consequence of a lack of scopolin biosynthesis,
307 as esculetin synthesis could occur *via* the demethylation of scopoletin [16]. Whether MYB10,
308 the closest homolog of MYB72, is also involved in coumarin biosynthesis has not yet been
309 established even if both genes are required for plant growth at alkaline pH [56]. It is
310 noteworthy that MYB72 and MYB10 are direct targets of URI and as a consequence, only
311 low levels of scopolin are detected in *uri* mutant roots [49].

312

313 Heavy metals availability also influences the expression of transcription factors involved in
314 the regulation of Fe homeostasis and thus coumarin biosynthesis [42]. For instance, the
315 expression of *PYE/bHLH47 (POPEYE)* and *MYB72* is induced in response to an excess of Zn,
316 Ni, Co and, to a lesser extent, Cd. In contrast, the expression of *FIT* is only induced in
317 response to an excess of Zn and Co.

318

319 In addition to the above-described TFs, MYB15, whose activity is necessary for the defense-
320 induced synthesis of guaiacyl lignin monomers, is proposed to regulate the basal synthesis of
321 scopoletin [57], whereas bHLH32 and PYE might participate to the inhibition of sideretin
322 glucosides levels under Fe deficiency and P sufficiency, respectively [47] (Figure 2). These
323 later observations suggest that *CYP82C4* could be a target of both bHLH32 and PYE. RNA
324 silencing is another mechanism that participates to the regulation of coumarin biosynthesis, in
325 particular the biosynthesis of scopolin in response to cold, osmotic stresses and high carbon
326 availability [12].

327

328 **Concluding remarks and future perspectives**

329 The secretion of coumarins into the rhizosphere under low Fe bioavailability conditions is now
330 considered as an essential component of the Fe-acquisition system of non-grass species. The
331 identification of fraxetin and sideretin as the main coumarins involved in Fe acquisition as
332 well as the key enzymes (i.e. F6'H1, COSY, S8H or CYP28C4) leading to their synthesis was
333 an important step forward in elucidating the coumarin biosynthetic pathway.

334

335 Nevertheless, there are still several steps related to coumarin conjugation, storage, transport
336 and trafficking that need to be elucidated and that would help in decrypting the precise mode
337 of action of coumarins involved in Fe acquisition in non-grass species. Understanding the
338 mechanisms by which this pathway is regulated at the transcriptional level by environmental
339 factors and in particular the availability of other macro- and micronutrients awaits further

340 investigations in order to integrate the complexity of the interactions between Fe and other
341 mineral nutrient homeostasis.

342

343 Since coumarin synthesis is a complex trait, the use of quantitative genetic approaches and in
344 particular GWAS (genome-wide association studies) might provide a powerful strategy to get
345 insights into the genetic basis of coumarins synthesis in plants either under Fe deficiency or
346 under combined stress conditions, and may help identifying missing molecular actors. For
347 instance, one might screen accessions by measuring the amount of different coumarins
348 present in roots or secreted into the surrounding media. Causal genes and alleles that will be
349 identified may have high potential for generating germplasm with increased Fe-assimilation
350 efficiency in soils where Fe availability is low but also in soils with contrasted mineral
351 nutrient composition and thus participate to the design of effective bio-fortification programs
352 and to combat global human Fe malnutrition (see also outstanding questions).

353

354

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501

502 GLOSSARY

503

504 **Aglycone:** the non-sugar component of a glycoside. In that form, a hydrogen atom replaces
505 the glycosyl group.

506 **Calcareous soil:** a soil that is rich in calcium carbonate and whose pH is alkaline (i.e. pH >
507 7).

508 **Coumarins:** secondary metabolites derived from the phenylpropanoid pathway notably
509 involved in plant/microbe interactions and plant iron acquisition.

510 **Flavonoids:** secondary metabolites derived from the phenylpropanoid pathway whose
511 biosynthesis in plants affects flower coloration, the response to various biotic (e.g. insects)
512 and abiotic stresses (e.g. U.V.) or the transport of hormones (i.e. auxin).

513 **Lignins:** secondary metabolites derived from the phenylpropanoid pathway providing
514 structural support and rigidity to plants and participating to the transport of water and the
515 defense against pathogens.

516 **Phenylpropanoids:** secondary metabolites derived from the amino acid phenylalanine.
517 Among the phenylpropanoid compounds are the lignins, the flavonoids and the coumarins.

518 **Rhizosphere:** part of the soil directly influenced by root secretions and associated
519 microorganisms.

520 **Trichoblasts:** specialized cells in root epidermis that develop into root hairs.

521 **2-4 D:** the 2,4-dichlorophenoxyacetic acid is a synthetic analog of the auxin plant hormone.
522

523 **FIGURE LEGENDS**

524

525 **Figure 1. Iron acquisition system in *Arabidopsis thaliana* roots.** Ferric Fe hydroxides
526 (FeOH₃) solubilization involves the secretion of protons (H⁺) into the rhizosphere by the P-
527 type ATPase AHA2. The acidification of the rhizosphere is necessary for the activity of
528 FRO2, which reduces Fe³⁺ into Fe²⁺. Fe²⁺ is then taken up into the root via the IRT1
529 transporter. AHA2, FRO2 and IRT1 form protein complexes at the plasma membrane of the
530 root epidermis. When Fe availability is low, the activity of AHA2, FRO2 and IRT1 is
531 induced. This process is accompanied by the production and secretion, via the
532 PDR9/ABCG37 transporter, of Fe-mobilizing coumarins. Once synthesized, coumarins are
533 glycosylated and stored into the vacuole. The molecular components involved in vacuolar
534 import and export of coumarins are still to be characterized. Prior to their secretion into the
535 rhizosphere, coumarins are deglycosylated. BGLU42 is responsible of scopolin
536 deglycosylation whereas the identity of the BGLUs involved in the deglycosylation of the Fe-
537 mobilizing coumarins remains elusive. It is hypothesized that once secreted, Fe-mobilizing
538 coumarins reduce Fe³⁺ into Fe²⁺, to make it available for IRT1 uptake. It is also hypothesized
539 that Fe³⁺-coumarin chelates might be directly taken up into the roots by a yet unknown
540 mechanism that could involve an epidermal transporter. This figure was created using
541 BioRender (<https://biorender.com/>).

542

543 **Figure 2. Coumarins biosynthesis pathway in *Arabidopsis thaliana*.** Plain arrows indicate
544 characterized enzymatic step, broken lines indicate uncharacterized step. Numbers refer to the
545 name of the different intermediate compounds synthesized during the biosynthesis of
546 scopoletin and esculetin, starting from phenylalanine. 1: L-phenylalanine, 2: cinnamic acid, 3:
547 coumarin, 4: *p*-coumaric acid, 5: *p*-coumaroyl-CoA, 6: 2-hydroxy-*p*-coumaroyl-CoA, 7:
548 umbelifereone, 8: caffeate, 9: caffeoyl-CoA, 10: 6 hydroxycaffeoyl-CoA, 11: ferrulate, 12:
549 feruloyl-CoA, 13: 6-hydroxyferuloyl-CoA. The biosynthesis of 9 from 5 is a three enzymatic
550 steps process and involves as intermediate the *p*-coumaroyl shikimate deriving from 5
551 (catalyzed by the HCT) and then the caffeoyl shikimate (catalyzed by the C3'H) form which
552 is synthesized 9 (catalyzed by the HCT). Capital letters indicate the enzymes involved in this
553 biosynthesis pathway. 4CL: 4-coumarate-CoA ligase, C3'H: *p*-coumarate 3-hydroxylase,
554 C4H: cinnamate 4-hydroxylase, CCoAOMT: caffeoyl-CoA 3-O-methyltransferase, COSY:
555 coumarin synthase, F6'H1: feruloyl-CoA 6'-hydroxylase 1, HCT: hydroxycinnamoyl-CoA
556 shikimate/quinate hydroxycinnamoyltransferase, PAL: phenylalanine ammonia-lyase.

557

558 **Figure 3. The iron-mobilizing coumarins biosynthesis pathway in *Arabidopsis thaliana***
559 **roots.** Fe-mobilizing coumarins are secondary metabolites displaying a catechol moiety (i.e.
560 two neighboring hydroxyl groups) and synthesized from feruloyl-CoA, an intermediate of the
561 phenylpropanoid pathway. The catechol moiety present on Fe-mobilizing coumarins is
562 necessary to interact with and mobilize the Fe present in soils. The FERULOYL-CoA 6'-
563 HYDROXYLASE 1 (F6'H1) catalyzes the first step committed to the biosynthesis of
564 coumarins, leading to the formation of 6'-hydroxyferuloyl-CoA. The conversion of 6'-
565 hydroxyferuloyl-CoA into scopoletin is partially spontaneous but the reaction efficiency is
566 significantly increased by the activity of COUMARIN SYNTHASE (COSY). Scopoletin is
567 then hydroxylated into fraxetin by the SCOPOLETIN 8-HYDROXYLASE (S8H). While
568 alkaline pH favors the biosynthesis of fraxetin, the main Fe-mobilizing coumarin secreted at
569 acidic pH is sideretin. Sideretin biosynthesis involves the activity of CYP82C4, a cytochrome
570 P450 enzyme. The biosynthesis of esculetin is still uncharacterized. This figure was created
571 using BioRender (<https://biorender.com/>).

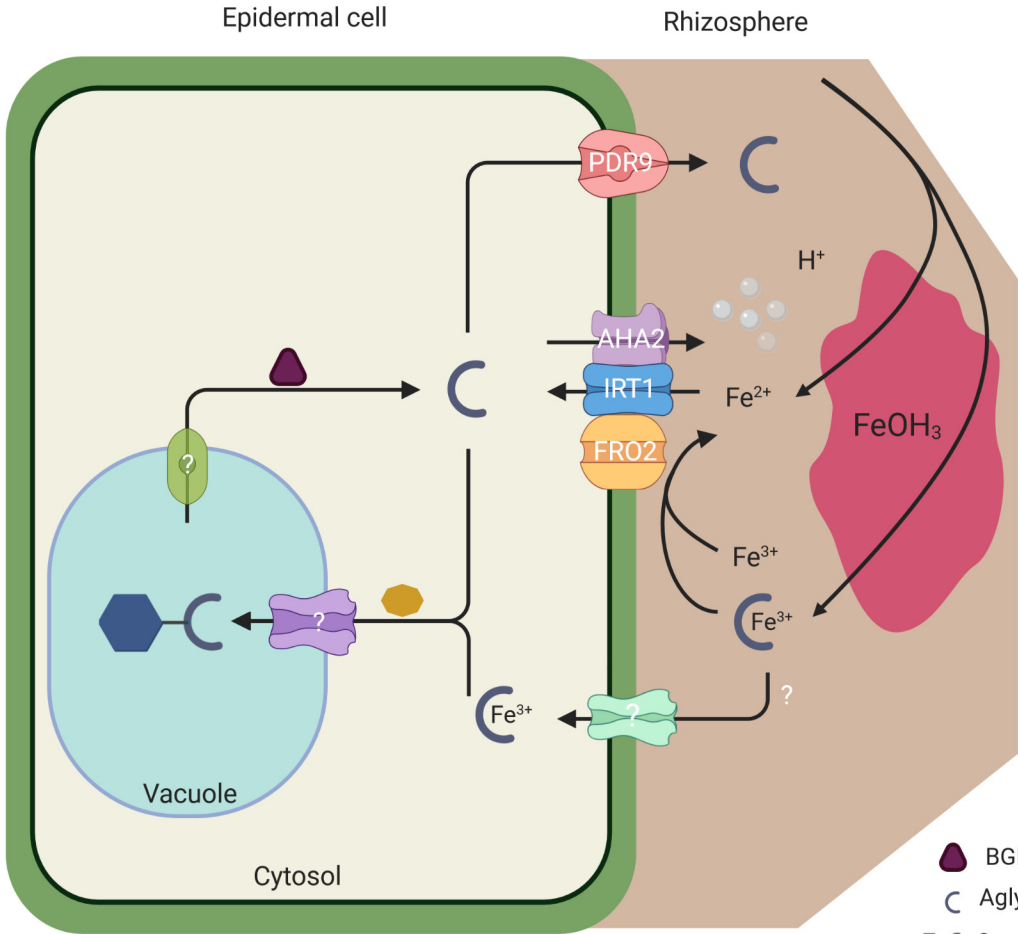
572





573 **Figure 4. Coumarins localization and dynamics in *Arabidopsis thaliana* roots.** Coumarins
574 (blue) are differentially accumulated and located in roots depending on Fe availability. Under
575 Fe sufficient condition, coumarins accumulate in the epidermis, the cortex and the
576 endodermis. Under Fe deficiency, coumarins accumulate at high level in the same cell types
577 except the trichoblasts (root hairs). Coumarins move from root to shoot (blue arrow) to
578 accumulate in shoot after Fe deficiency, cold stress or pathogen infections. Glycosylated
579 coumarins are able to move via the phloem to reach the root once exogenously applied on
580 cotyledons surface (blue arrow). PDR9 is located at the epidermis, allowing coumarins
581 secretion into the rhizosphere. In response to Fe deficiency, PDR9 accumulation increases in
582 this tissue and in the cortex allowing coumarins movement from the cortex cell layer to the
583 rhizosphere. Promoter activities and protein localizations of biosynthetic enzymes are
584 indicated in light red and green squares, respectively. Several transporters involved in
585 coumarins cell-to-cell movement are still to be identified. ep, epidermis; c, cortex; e,
586 endodermis; s, stele. This figure was created using BioRender (<https://biorender.com/>).

587

588 **Figure 5. Transcriptional regulation of coumarins biosynthesis in *Arabidopsis thaliana***
589 **roots.** A complex regulatory network controls the Fe deficiency responses. When Fe
590 availability is scarce, URI/bHLH121 and clade IVc bHLH transcription factors (i.e. bHLH34,

591 bHLH104, bHLH115 and ILR3/bHLH115) heterodimerize to activate the expression of
592 several regulatory genes involved in the control of Fe homeostasis. It includes genes encoding
593 the E3-ubiquitin ligases BRUTUS (BTS) and BRUTUS LIKE 1 (BTSL1), the IRONMAN
594 peptides (IMAs), the MYB72, MYB10 and PYE/bHLH47 transcription factors. Clade Ib
595 bHLH (i.e. bHLH38, bHLH39, bHLH100 and bHLH101) are also among the targets.
596 FIT/bHLH27 expression is induced in response to Fe deficiency. FIT interacts with clade Ib
597 bHLH to activate the expression of genes involved in Fe uptake (e.g. *FRO2* and *IRT1*) and
598 coumarins biosynthesis (i.e. *S8H* and *CYP82C4*). The expression of *F6'H1*, *BGLU42* and
599 *PDR9* is under the control of both the FIT/clade Ib complexes and the MYB72 transcription
600 factor (the involvement of its closets homolog, MYB10, is still to be demonstrated). The
601 MYB72-dependent regulation of *F6'H1*, *BGLU42* and *PDR9* most probably allows
602 mobilizing coumarins for both Fe acquisition (together with *FRO2* and *IRT1*) and to modulate
603 the plant/microorganism interactions. Concomitantly, ILR3 and PYE heterodimers inhibit the
604 expression of genes involved in Fe transport via the phloem (e.g. *NAS4*) and Fe storage (e.g.
605 *FER1*). URI and FIT activities are regulated at the posttranslational level by phosphorylation
606 (i.e. active when phosphorylated). The direct interaction of clade IVa bHLHs (i.e. bHLH18,
607 bHLH19, bHLH20 and bHLH25) with FIT promotes FIT degradation via the 26S proteasome
608 pathway. In addition, MYB15 participates to the basal expression level of *F6'H1* and PYE
609 and bHLH32 repress *CYP82C4* and *S8H* expression in response to phosphate deficiency.
610 bHLH32 also repress *CYP82C4* expression under Fe deficiency conditions. *: FIT expression
611 is partly dependent on URI activity. Transcription factors labeled in black, red and white
612 modulate the expression of their target genes in Fe deficiency, phosphate deficiency or control
613 condition, respectively. This figure was created using BioRender (<https://biorender.com/>).
614



-  BGLUs
-  Aglycone coumarins
-  Coumarins glucoside
-  Glycosyltransferase

Shikimate pathway

