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

3  
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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 CAFFEOYL-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 esculin 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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500 1926.

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 (FeOH<sub>3</sub>) solubilization involves the secretion of protons (H<sup>+</sup>) 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 Fe<sup>3+</sup> into Fe<sup>2+</sup>. Fe<sup>2+</sup> 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 Fe<sup>3+</sup> into Fe<sup>2+</sup>, to make it available for IRT1 uptake. It is also hypothesized  
539 that Fe<sup>3+</sup>-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/>).

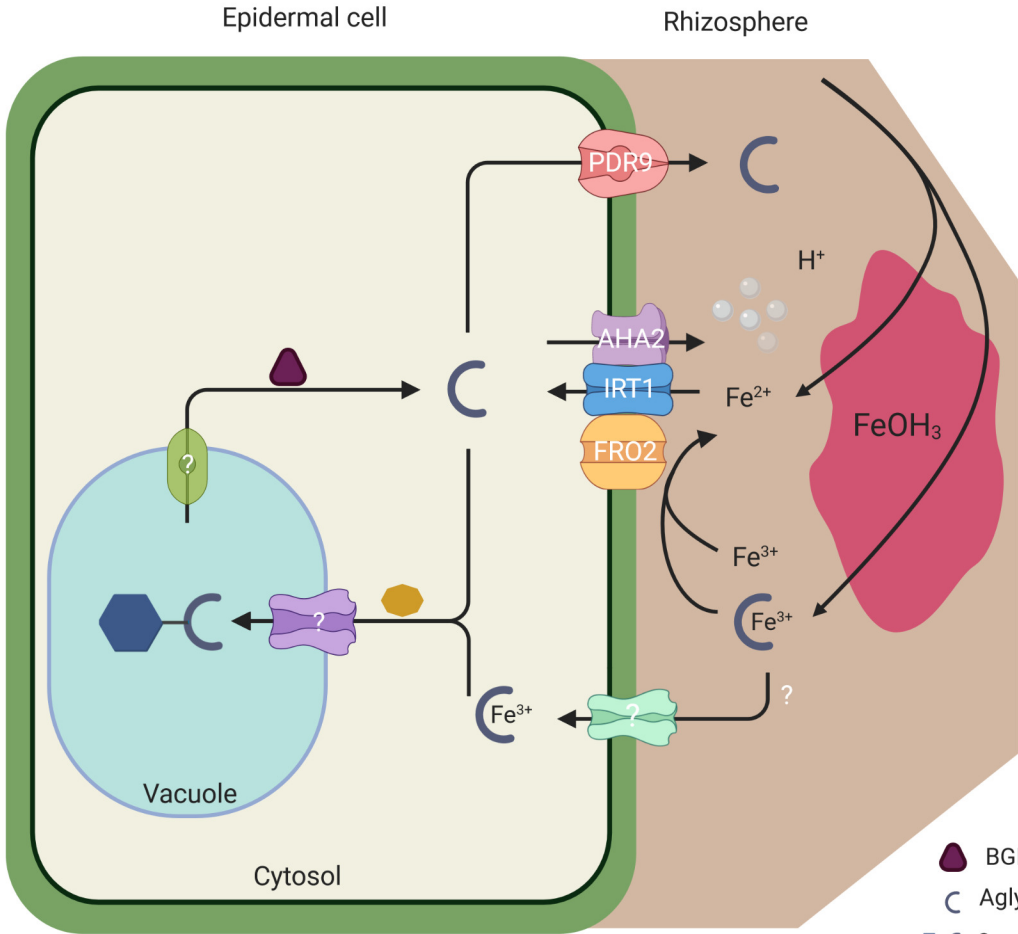
572





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

