

1 Disruption of the rice 4-DEOXYOROBANCHOL HYDROXYLASE 2 unravels specific functions of canonical strigolactones

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19 **Abstract:**

20 Strigolactones (SLs) regulate many developmental processes, including shoot-
21 branching/tillering, and mediate rhizospheric interactions. SLs are structurally diverse,
22 divided into a canonical and a non-canonical sub-family. To better understand the
23 biological function of particular SLs, we generated CRISPR/Cas9 mutants disrupted in
24 *OsMAX1-1400* or *OsMAX1-1900*, which encode cytochrome P450 enzymes (CYP711A
25 clade) contributing to SL diversity. The disruption of *OsMAX1-1900* did neither affect the
26 SL pattern nor plant architecture, indicating a functional redundancy. In contrast,
27 disruption of *OsMAX1-1400* activity, a 4-deoxyorobanchol hydroxylase, led to a
28 complete lack of orobanchol and an accumulation of its precursor 4-deoxyorobanchol
29 (4DO), both of which are a canonical SLs common in different plant species,
30 accompanied by higher levels of the non-canonical methyl 4-oxo-carlactonoate (4-oxo-
31 MeCLA). *Os1400* mutants showed also shorter plant height, panicle and panicle base
32 length, but did not exhibit a tillering phenotype. Hormone quantification and
33 transcriptome analysis revealed elevated auxin levels and changes in the expression of

34 auxin-related, as well as of SL biosynthetic genes. Interestingly, the *Os900/1400* double
35 mutant lacking both orobanchol and 4DO did not show the observed *Os1400*
36 architectural phenotypes, indicating that they are a result of 4DO accumulation. A
37 comparison of the mycorrhization and *Striga* seed germinating activity of *Os900*,
38 *Os900/1400*, and *Os1400* loss-of-function mutants demonstrates that the germination
39 activity positively correlates with 4DO content while disrupting *OsMAX1-1400* negatively
40 impact mycorrhizal symbiosis. Taken together, our paper deciphers the biological
41 function of canonical SLs in rice and depicts their particular contributions to establishing
42 architecture and rhizospheric communications.

43

44 **Key words:** Strigolactones, Cytochrome P450, Plant Architecture, *Striga*, Arbuscular
45 mycorrhizal fungi

46

47 **Introduction**

48 The apocarotenoid-derived strigolactones (SLs) are a novel class of plant hormones
49 induced under low phosphate (Pi) condition that inhibits shoot branching/tillering
50 (Gomez-Roldan et al., 2008; Umehara et al., 2008) and regulates other plant processes
51 and features, including root development, stem thickness, and leaf senescence (Al-
52 Babili & Bouwmeester, 2015; Fiorilli et al., 2019). Before being recognized as a plant
53 hormone, SLs were first discovered to be the germinating stimulants for root parasitic
54 weeds, such as *Orobanche* and *Striga spp.* (Cook et al., 1966), and later found as an
55 initiation signal for establishing beneficial arbuscular mycorrhizal fungi (AMF) symbiosis,
56 by inducing the AMF hyphal branching (Akiyama et al., 2005; Lanfranco et al., 2018). In
57 addition, SLs can orchestrate plant architecture as SL-deficient plants, such as rice *d17*,
58 consistently exhibit a distinct phenotype comprising higher numbers of branches/tillers,
59 shorter shoot and primary root length when compared to wild-type (WT) (Al-Babili &
60 Bouwmeester, 2015; Gomez-Roldan et al., 2008; Morris et al., 2001), suggesting that
61 SLs are more than rhizospheric signals (Wang et al., 2022a).

62

63 SLs are characterized based on their exclusive chemical structure, the lactone ring (D-
64 ring; Fig.1A). The latter is linked to an enol-ether bridge, essential for the SL biological

65 activity (Yoneyama et al., 2018). According to the presence or absence of the BC-ring
66 (Fig.1A), they are classified into canonical and non-canonical SLs, respectively (Al-
67 Bibili & Bouwmeester, 2015; Wang et al., 2021). In general, the evolutionarily-
68 conserved SL biosynthetic pathway in land plants starts specifically with 9-cis- β -
69 carotene after its isomerization by DWARF27 (D27) (Abuauf et al., 2018). Then, two
70 carotenoid cleavage dioxygenases (CCDs), CCD7 (D17) and CCD8 (D10), cleave
71 successively 9-cis- β -carotene into carlactone (CL), the core intermediate of SL
72 biosynthesis *in planta* (Alder et al., 2012; Bruno et al., 2014; Chen et al., 2022; Seto et
73 al., 2014; Wang et al., 2021).

74

75 Until now, more than 35 natural SLs, with different chemical structures, are identified *in*
76 *Plantae* (Yoneyama et al., 2018). Their structural diversity arose from the CL catabolism
77 by MORE AXILLARY GROWTH1 (MAX1) from the cytochrome P450 monooxygenase
78 (CYP) 711A family (Booker et al., 2005; Cardoso et al., 2014; Lazar & Goodman, 2006),
79 and the recently identified CYP722C, CYP712G1, CYP706C37 clades (Li et al., 2023;
80 Wakabayashi et al., 2019; Wang et al., 2022c). In rice, OsMAX1-900 repeatedly
81 oxygenates CL to produce the canonical SL 4-deoxyorobanchol (4DO) *in vivo* (Ito et al.,
82 2022). Based on *in vitro* studies and expression in *Nicotiana benthamiana*, 4DO is
83 further hydroxylated into orobanchol (Oro) by another CYP711A enzyme, OsMAX1-
84 1400, (Zhang et al., 2014).

85

86 The biological relevance of such a big family of compounds is yet to be discovered by
87 the scientific community. Therefore, we started our investigation in rice and created
88 CRISPR-mediated mutant lines of OsMAX1s. Our previous study surprisingly showed
89 that mutants created by targeting OSMAX1-900 do not display a typical SL-deficiency
90 phenotype (Ito et al., 2022). This revealed that canonical SLs are not the major tillering
91 regulators, and hence the biological functions of canonical SLs inside plants remain
92 elusive. To have a comprehensive view, in this work we aimed to understand the
93 biological roles of canonical SLs in rice by studying mutants defective of *Osmax1-1400*,
94 the other rice enzyme involved in canonical SLs synthesis.

95

96 **Results and Discussion**

97 **Characterization of rice *MAX1* homologs**

98 To investigate the biological functions of canonical SLs in rice, we generated several
99 mutant lines using CRISPR/Cas9 technology. We targeted the biosynthesis of canonical
100 SLs by generating biallelic homozygote *Osmax1-1400* (*Os1400-12SIII* and *-12SIV*, Fig.
101 S2). We also generated *Osmax1-1900* (*Os1900-13DI* and *-13EI*, Fig. S3) rice mutant
102 lines to understand whether the *MAX1-1900* would contribute to SL biosynthesis *in*
103 *planta*, as *MAX1-1900* is phylogenetically from a distinct clade, and only shown to
104 weakly convert CL into CLA *in vitro* (Marzec et al., 2020; Yoneyama et al., 2018).

105

106 First, we quantified the known SLs in these mutants' roots and root exudates, together
107 with *Os900* and SL-deficient *d17*, using Liquid Chromatography Tandem-Mass
108 Spectrometry (LC-MS/MS) under phosphate (Pi) starvation conditions (Fig. 1C; Fig. 1D;
109 Fig. S4). As expected, no SLs were detected in *d17*, while the CRISPR/Cas9-induced
110 deletion impaired the biosynthesis of the canonical SLs, which was evidenced by the
111 absent Oro in the roots and root exudates of *Os1400* mutants (Fig. 1C). This confirmed
112 *OsMAX1-1400* as the 4DO hydroxylase *in planta* (Zhang et al., 2014). Interestingly, the
113 accumulation of two SLs, 4DO and the putative methyl 4-oxo-carlactonoate (4-oxo-
114 MeCLA), produced through *OsMAX1-900* (Ito et al., 2022), were doubled in the root
115 exudates of *Os1400* when compared to wild-type (WT) plants (Fig. 1D). The production
116 of 4-oxo-MeCLA is likely through an additional step by an uncharacterized
117 methyltransferase (Ito et al., 2022), suggesting that 4DO metabolism is exclusive by
118 *OsMAX1-1400*. Moreover, the amount of putative non-canonical SLs, CL+30 and oxo-
119 CL (Ito et al., 2022; Wang et al., 2022a), in the root exudates of *Os1400* were
120 comparable to that of WT (Fig. S4), affirming the mutation of *Os1400* mainly affects the
121 canonical SL Oro metabolism.

122

123 In contrast, we did not observe remarkable changes in SL composition in the root
124 tissues and root exudates between *Os1900* mutants and WT (Fig. S5), indicating that
125 the role of *OsMAX1-1900* might be functionally redundant in SL biosynthesis in plants.
126 This was supported by no consistently significant differences in the shoot and root

127 phenotypes grown under normal or low Pi conditions as well as *Striga* germination
128 activity, when compared to the WT (Fig. S6, Fig. S7).

129

130 **Accumulation of 4-deoxyorobanchol negatively regulates rice plant growth and**
131 **development**

132 Recently, we reported that rice lacking canonical SLs, 4DO and Oro, displays shoot
133 phenotypes comparable to WT, revealing that canonical SLs are not the major players
134 in regulating shoot architectures (Ito et al., 2022). However, rice has duplicated *MAX1*
135 genes during evolution (Marzec et al., 2020); intriguingly, *OsMAX1-1400* is the only rice
136 *MAX1* acting in the final step of canonical SL biosynthesis (Fig. 1A) (Al-Babili &
137 Bouwmeester, 2015; Zhang et al., 2014). Therefore, we suspected that *Os1400* and its
138 downstream metabolite Oro might hold biological importance in plants. To test this
139 hypothesis, we phenotyped *Os1400*, *Os900*, *d17*, and WT rice under soil and
140 hydroponic conditions. Expectedly, the *MAX1* mutants, grown in greenhouse, did not
141 showed high tiller numbers with dwarf appearance, the typical SL-deficit (*d17*)
142 phenotype (Fig. 2A). Instead, the plant height, panicle length, and length of the panicle
143 base (the distance from flag leaf auricle to the panicle base on the primary branch) of
144 *Os1400* mutants were significantly shorter than that of WT and *Os900* (Fig. 2B). No
145 differences were observed in the total number of tillers, productive tillers, and average
146 panicle numbers under greenhouse conditions (Fig. S8). The shorter shoot phenotype
147 was also detected in the hydroponically grown *Os1400* mutants under both normal and
148 low-Pi conditions (Fig. S9; Fig. S10).

149

150 It seems that the accumulation of 4DO could be an endogenous unfavorable/toxic signal
151 to rice plants. To confirm the assumption that the shorter shoot phenotype is caused by
152 4DO accumulation, we exogenously supplied 4DO at 300 nM and 900 nM under normal
153 growth conditions -without triggering SL biosynthesis- to WT and *Os1400* mutants.
154 Interestingly, 300 nM 4DO treatment remarkably increased the difference of shoot
155 length in *Os1400* mutants compared to untreated WT; however, with 900 nM 4DO
156 application, the shoot length in the WT was also suppressed, suggesting that 4DO is a
157 negative regulator of shoot growth and development in rice (Fig. S11). On the other

158 hand, we observed a longer root length tendency in *Os1400* than WT (Fig. S10) under
159 low-Pi conditions, while a decreased tendency in root length and root diameter under
160 normal conditions (Fig. S9; Fig. S12). As SL biosynthesis is triggered by Pi-starvation,
161 we postulated that Pi content might influence the amount of 4DO accumulated in the
162 plant, leading to the different tendency in root length under various growth conditions.
163 Indeed, exogenous 4DO application under normal conditions, mimicking SL
164 biosynthesis, enhanced the root length in all treated rice plants grown in hydroponics
165 (Fig. S11).

166

167 Next, to check our rescue hypothesis by reducing the level of 4DO accumulated in
168 *Os1400* mutants, we treated *Os1400* grown under normal conditions with 5 μ M TIS108,
169 a MAX1-900 and MAX1-1400 inhibitor (Ito et al., 2022). The root length and crown root
170 numbers of mutants treated with TIS108 were restored to that of the WT (Fig. S13),
171 providing a further evidence that the enzymatic activity on 4DO by OsMAX1-1400 is
172 important for an optimal rice root development. In fact, the root-released 4DO level of
173 *Os1400* mutants was suppressed upon TIS108 treatment, accompanied by the
174 accumulation of the putative non-canonical SLs, CL+30 and oxo-CL (Fig. S14) (Ito et
175 al., 2022; Wang et al., 2022a). Although the shoot length of *Os1400* mutants no longer
176 showed difference to the WT when treated with TIS108, we observed that two-week
177 application of TIS108 unexpectedly decreased the shoot length of WT (Fig. S13),
178 probably due to unspecific compound effects on other CYPs. Hence, to specifically
179 understand the biological roles of canonical SL biosynthesis, we generated a
180 homozygous *Osmax1-900/1400 (Os900/1400-4B)* rice double mutant line (Fig. S15),
181 which has full disruption of canonical SLs, to compare with the TIS108 observation.
182 Notably, the observed phenotypes of *Os1400* mutants were no longer present in the
183 double mutant in all growth conditions (Fig. 2; Fig. S8; Fig. S9; Fig. S10; Fig. S12),
184 together with the absence of canonical SLs and the accumulation of non-canonical SLs
185 in the roots and root exudates of *Os900/1400* (Fig. 1C; Fig. 1D; Fig. S4). Sharing the
186 same rescue mechanism as TIS treatment, suppressing both 4DO and Oro
187 biosynthesis, *Os900/1400* provides a stronger genetic evidence without the possibilities

188 of chemical effects. These suggest that the metabolism of 4DO by OsMAX1-1400 is
189 required for normal rice growth and development.

190

191 One previous study had contradictory results, showing a high-tillering phenotype with
192 low canonical SLs in the Bala rice that has both a deletion of *MAX1-900* and *MAX1-*
193 *1400* (Cardoso et al., 2014). However, our observations hold stronger genetic evidence
194 since these genetic materials were compared within the same cultivar, while the tillering
195 phenotype of Bala rice might be a consequence of other genetic regulations. Notably, 4-
196 oxo-MeCLA was present in the root exudate of *Os900/1400*, as well as in Bala rice root
197 exudate (Cardoso et al., 2014), indicating that the biosynthesis of this non-canonical SL
198 is independent of *MAX1-900* and *MAX1-1400*. Overall, we can conclude that 4DO and
199 Oro are not tillering-inhibitory regulators, but their unbalanced metabolism negatively
200 affects the physiological development of rice plants.

201

202 Furthermore, we investigated the rice transcriptome of the *Os1400* grown under normal
203 and low-Pi conditions compared to WT by RNAseq (Data S1). In the differentially
204 expressed genes (DEGs), there were 1712 upregulated genes and 1465 downregulated
205 genes under normal conditions, while 5890 upregulated genes and 3569 downregulated
206 genes were observed under low-Pi conditions (Fig. S16). None of the DEGs related to
207 tillering or SL biosynthesis was observed under normal conditions (Tables S1); in
208 contrast, the transcripts of SL biosynthesis and signaling were generally downregulated
209 under low-Pi conditions (Fig. 3A, Tables S2), suggesting that the 4DO accumulation
210 might lead to a negative feedback loop in *Os1400* mutants. Interestingly, under both
211 conditions, we observed many downregulated auxin-related genes in *Os1400* mutants
212 (Fig. 3B, Tables S1-S2).

213

214 Auxin regulates meristem activities and interplays with SLs on the root and shoot
215 development (Su et al., 2011; Xiao et al., 2019; Yang et al., 2019); thus, we
216 hypothesized the phenotypes observed in *Os1400* mutants could be linked to auxin. We
217 then determined the hormone content of auxin (IAA), gibberellin (GA), abscisic acid
218 (ABA), salicylic acid (SA), and jasmonic acid (JA) in roots and shoot bases (root-shoot

219 junction) of *Os1400* mutants under normal and low Pi conditions. We did not detect any
220 consistent significant difference in the levels of GAs, ABA, SA, or JA (Fig. S17), but
221 observed a remarkable increase of IAA level in root and shoot bases in both growth
222 conditions, compared to the WT (Fig. 3C; Fig. S17). Consistently, excess of IAA
223 content, by either overexpression of *OsPIN2* or exogenous IAA application, causes
224 shorter shoot phenotype in rice (Liu et al., 2019; Sun et al., 2019). Furthermore, we
225 performed 5-Ethynyl-2'-deoxyuridine (EdU) staining to visualize proliferating cells and
226 measured the root meristem length. Yet, no clear differences were observed between
227 *Os1400* mutants and WT (Fig. S18). Accordingly, we demonstrated that the shoot
228 phenotypes of *Os1400* plants are likely due to auxin homeostasis modulation, but the
229 role of auxin in *Os1400* roots needs further investigation.

230

231 **The metabolism of rice canonical SL, 4-deoxyorobanchol, is associated to 232 rhizospheric signals**

233 Although all SLs seem to be communicating signals in the rhizosphere (Ito et al., 2022;
234 Wang et al., 2022a), their bioactivities - to induce AM hyphal branching and trigger
235 parasitic seed germination - depend largely on their chemical structures (Gobena et al.,
236 2017; Mori et al., 2016). The distinguishable root-released SL compositions in *Os900*,
237 *Os900/1400*, and *Os1400* (Fig. 1; Fig. S1) make them good candidates to investigate
238 the possible functions of these metabolites in the rhizosphere. We then tested the
239 germination activity of *Os900*, *Os1400*, and *Os900/1400* root exudates on *Striga*
240 *hermonthica* seeds. Compared to WT exudates, we observed more than 40% decrease
241 in the *Striga* germination of *Os900* exudates and an even lower germination rate of
242 *Os900/1400* exudates (Fig. 4A), indicating that 4-oxo-MeCLA is not a predominate
243 germinating signal for *Striga* (Fig. S4). Although the stimulation activity on *Striga*
244 germination of *Os1400* exudates was comparable to the WT at 1:1 dilution, we found an
245 increased tendency at 1:3 dilution (Fig. 4A; Fig. S19A). This reveals that 4DO is a
246 stronger germination cue for *Striga* seeds than Oro, supported experimentally by 10 μ M
247 Oro exerting less seed germination activity than 1 μ M *rac*-GR24, a 4DO-like SL analog
248 (Fig. S19B).

249

250 Moreover, Oro seems to be the preferable signal for AM symbiosis (Mori et al., 2016),
251 we then investigated the role of 4DO and Oro in establishing AM symbiosis by
252 comparing Os900, Os1400, Os900/1400, and WT. For this purpose, we examined the
253 root colonization with the AMF *Rhizophagus irregularis* after 10- and 40-days post
254 inoculation (dpi) and measured the transcript level of *OsPT11*, a specific AM inducible
255 Pi-transporter gene (Güimil et al., 2005). At 10 dpi, there was a delay in colonization of
256 all mutant roots compared to WT roots; whereas, at 40 dpi, the colonization of Os1400
257 and Os900/1400 mutants was surprisingly much lower than that of the WT and Os900
258 (Fig. 4B; Fig. S20), which indicates that *MAX1-1400* is crucial for maintaining AM
259 colonization. Additionally, absence of *MAX1-1400* did not influence the intraradical
260 fungal structures of the arbuscules. Instead, they appeared well developed and
261 regularly branched (Fig. 4C), suggesting that *MAX1-1400* does not affect the fungal
262 morphology. Therefore, we can speculate that *MAX1* duplication is highly associated
263 with AM symbiosis; besides, the decreased colonization level of Os900/1400 mutant
264 further supported that the reduced AM symbiotic pattern upon TIS108 application in WT
265 plants (Ito et al., 2022) might be a consequence of *MAX1-1400* suppression.

266

267 Taken together, the generation of the CRISPR-mediated Os1400 rice mutants allowed
268 us to reveal the biological roles of canonical SLs in plant growth and development, and
269 as well confirmed OsMAX1-1400 as the 4DO hydroxylase *in planta*. Finally, we can
270 conclude that the canonical SLs, 4DO and Oro, are critical rhizospheric signals for the
271 interaction with AMF and root parasitic plants, and the *MAX1* duplication might be also
272 evolutionary required for beneficial symbiosis in rice. Importantly, knocking out entire
273 canonical SLs, without damaging plant architecture in the Os900/1400 double mutant,
274 seems promising to reduce the yield loss caused by *Striga* and other root parasitic
275 plants; thus, offering an alternative way to improve global food security.

276

277 **Material and Methods**

278 **Plant material and growth conditions**

279 *Oryza sativa* Nipponbare d17 (Butt et al., 2018), *max1-900* (Ito et al., 2022), *max1-*
280 *1400*, *max1-900/1400*, *max1-1900*, and WT rice plants were grown under controlled

281 conditions (a 12 h photoperiod, 200- μ mol photons $m^{-2} s^{-1}$ and day/night temperature of
282 27/25 °C). All rice seeds were first surface-sterilized in a 50% sodium hypochlorite
283 solution with 0.01 % Tween-20 for 15 min, then rinsed with sterile water, before being
284 germinated in the dark overnight. The pre-germinated seeds were placed on Petri
285 dishes containing half-strength liquid Murashige and Skoog (MS) medium and
286 incubated in a growth chamber for 7 days. Thereafter, the seedlings were transferred
287 into 50mL black falcon tubes filled with half-strength modified Hoagland nutrient solution
288 with adjusted pH to 5.8. The nutrient solution consisted of 5.6 mM NH_4NO_3 , 0.8 mM
289 $MgSO_4 \cdot 7H_2O$, 0.8 mM K_2SO_4 , 0.18 mM $FeSO_4 \cdot 7H_2O$, 0.18 mM $Na_2EDTA \cdot 2H_2O$, 1.6 mM
290 $CaCl_2 \cdot 2H_2O$, 0.8 mM KNO_3 , 0.023 mM H_3BO_3 , 0.0045 mM $MnCl_2 \cdot 4H_2O$, 0.0003 mM
291 $CuSO_4 \cdot 5H_2O$, 0.0015 mM $ZnCl_2$, 0.0001 mM $Na_2MoO_4 \cdot 2H_2O$ and 0.4 mM $K_2HPO_4 \cdot 2H_2O$.
292

293 **Generation of Os1900, Os1400, and Os1400/900 plants**

294 Two guide RNAs [gRNAs; single gRNA3 (sgRNA3), 5' -tgcgAACAGGTTGAAATTGG-3' and
295 sgRNA4, 5' -ctcgAGTTCACTACTCGAT-3'] were designed to target the rice (*O. sativa* L. ssp.
296 *japonica* cv. *Nipponbare*) *OsMAX1-1400* (*Os01g0701400* /AP014957) gene. By using
297 Golden Gate cloning, the tRNA-gRNA-Cas9 cassette was assembly into the pRGEB32
298 binary vector that has hygromycin resistance gene for selection. With mature seeds,
299 *Nipponbare* calli were induced and transformed with *Agrobacterium tumefaciens*
300 EHA105 culture containing the plasmid of interest. Later, shoots and roots were
301 regenerated in a Percival growth chamber (CLF Plant Climatics GmbH, model CU
302 36L5), and then transferred to soil and grown in a greenhouse at 28°C day/22°C night.
303

304 Genomic DNA was extracted from the rice young leaves, and plant transgenicity and
305 mutagenicity were demonstrated. Through polymerase chain reaction (PCR)
306 amplification, the transgenic plants were recognized when the pRGEB32-specific
307 primers, pRGEB32-F (5'-ccacgtatgtgaagaagataaaactg-3') and pRGEB32-R (5'-
308 gataggTTAAGGGTATCCAAATTGAGAC-3'), bind to the surrounding region of the insertion sites
309 in the pRGEB32 vector. For identifying CRISPR-mediated mutations, the DNA region
310 that includes the sgRNA target sites were amplified using genome specific primers

311 Os1400 sg3-sg4 F (5'-tcagcgcgctcacttacga -3') and Os1400 sg4 F1 (5'-
312 atcccaagaacttcccggag-3').

313

314

315 **Hydroponic culture of rice seedlings**

316 The hydroponic culture system is built with 50-mL black falcon tubes with punctured
317 caps inserted with a 1.5-ml bottomless Eppendorf tube in the center. Nutrition solution,
318 containing normal (+Pi) or low 0.004 mM K₂HPO₄·3H₂O (lowPi), was applied to the
319 transferred 1-week old seedlings for the following 2 weeks. The solutions were changed
320 every 3 days, and adjusted to pH 5.8 every time before applying. All plants were kept in
321 the solution for 3 weeks, except the plants for 4DO application and EdU staining were
322 10-days seedlings.

323

324 **Phenotyping in pots under greenhouse conditions**

325 To study the phenotype of Os1400 and Os900/1400 mutants, seedlings were
326 transferred into pots packed with soil. The soil were soaked with half-strength modified
327 Hoagland nutrient solution in advance. The nutrient solution comprised 5.6 mM
328 NH₄NO₃, 0.8 mM MgSO₄·7H₂O, 0.8 mM K₂SO₄, 0.18 mM FeSO₄·7H₂O, 0.18 mM
329 Na₂EDTA·2H₂O, 1.6 mM CaCl₂·2H₂O, 0.8 mM KNO₃, 0.023 mM H₃BO₃, 0.0045 mM
330 MnCl₂·4H₂O, 0.0003 mM CuSO₄·5H₂O, 0.0015 mM ZnCl₂, 0.0001 mM
331 Na₂MoO₄·2H₂O, and 0.4 mM K₂HPO₄·2H₂O. The pH of the solution was adjusted to
332 5.8, and the solution was applied every third day. On day 120, phenotypic data were
333 recorded. The plants were grown in a greenhouse from February to May 2022, in
334 Thuwal (Saudi Arabia).

335

336 **Exogenous applications of 4DO and TIS108**

337 For investigating the effect of 4DO (Olchemim, Czech Republic) on different genotypes,
338 1-week-old seedlings were grown hydroponically in half-strength Hoagland nutrient
339 solution containing 0.4 mM K₂HPO₄·2H₂O (+Pi), 300 nM or 900 nM 4DO (dissolved in
340 acetone), or the corresponding volume of the solvent (mock; acetone) for 10 or 14 days.
341 The solution was changed three times per week, adding the chemical at each renewal.

342

343 For investigating the effect of TIS108, 2-week-old rice seedlings were grown
344 hydroponically in half-strength Hoagland nutrient solution containing 0.4 mM
345 $K_2HPO_4 \cdot 2H_2O$ (+Pi), 5 μM TIS108 (dissolved in acetone), or the corresponding volume
346 of the solvent (mock; acetone) for 14 days. The solution was changed twice per week,
347 adding the chemical at each renewal.

348

349 **SL quantification in root tissues and exudates**

350 Analysis of SLs in rice root exudates was performed according to the published protocol
351 (Wang et al., 2022b). Briefly, root exudates spiked with 2 ng of GR24 were brought on a
352 C₁₈-Fast Reversed-SPE column (500 mg/3 mL), preconditioned with 3 mL of methanol
353 and followed with 3 mL of water. After washing with 3 mL of water, SLs were eluted with
354 5 mL of acetone. Thereafter, SLs-containing fraction was concentrated to SL aqueous
355 solution (~500 μL), followed by 1 mL of ethyl acetate extraction. 750 μL of SL enriched
356 fraction was dried under vacuum. The final extract was re-dissolved in 100 μL of
357 acetonitrile: water (25:75, v:v) and filtered through a 0.22 μm filter for LC-MS/MS
358 analysis.

359

360 SL extraction from root tissues was followed the procedure (Wang et al., 2019). Around
361 25 mg of lyophilized and grinded rice root tissues, spiked with 2 ng of GR24, were
362 extracted twice with 2 mL of ethyl acetate in an ultrasound bath (Branson 3510
363 ultrasonic bath) for 15 min, followed by centrifugation for 8 min at 3800 rpm at 4 °C. The
364 two supernatants were combined and dried under vacuum. The residue was dissolved
365 in 50 μL of ethyl acetate and 2 mL of normal hexane, purifying with a Silica Cartridges
366 SPE column (500 mg/3 mL). After washing with 3 mL of hexane, SLs were eluted in
367 3 mL of ethyl acetate and evaporated to dryness under vacuum. The final extract was
368 re-dissolved in 150 μL of acetonitrile: water (25:75, v:v) and filtered through a 0.22 μm
369 filter for LC-MS/MS analysis.

370

371 SLs were quantified by LC-MS/MS using UHPLC-Triple-Stage Quadrupole Mass
372 Spectrometer (Thermo Scientific™ Altis™). Chromatographic separation was achieved

373 on the Hypersil GOLD C₁₈ Selectivity HPLC Columns (150 × 4.6 mm; 3 µm; Thermo
374 Scientific™) with mobile phases consisting of water (A) and acetonitrile (B), both
375 containing 0.1% formic acid, and the following linear gradient (flow rate, 0.5 mL/min): 0–
376 15 min, 25%–100 % B, followed by washing with 100 % B and equilibration with 25 % B
377 for 3 min. The injection volume was 10 µL, and the column temperature was maintained
378 at 35 °C for each run. The MS parameters of Thermo Scientific™ Altis™ were as
379 follows: positive ion mode, ion source of H-ESI, ion spray voltage of 5000 V, sheath gas
380 of 40 arbitrary units, aux gas of 15 arbitrary units, sweep gas of 20 arbitrary units, ion
381 transfer tube gas temperature of 350 °C, vaporizer temperature of 350 °C, collision
382 energy of 17 eV, CID gas of 2 mTorr, and full width at half maximum (FWHM) 0.2 Da of
383 Q1/Q3 mass. The characteristic Multiple Reaction Monitoring (MRM) transitions
384 (precursor ion → product ion) were 331.15→216.0, 331.15→234.1, 331.15→97.02 for
385 4-deoxyorobanchol; 347.14→329.14, 347.14→233.12, 347.14→ 205.12, 347.14→97.02
386 for orobanchol; 361.16→ 247.12, 361.16→177.05, 361.16→208.07, 361.16→97.02 for
387 putative 4-oxo-MeCLA; 333.17→219.2, 333.17→173.2, 333.17→201.2, 333.17→97.02
388 for putative 4-oxo-hydroxyl-CL (CL+30); 317.17→164.08, 317.17→97.02 for putative
389 oxo-CL (CL+14); 299.09→158.06, 299.09→157.06, 299.09→97.02 for GR24.
390

391 **Quantification of plant hormones**

392 Quantification of endogenous hormones was followed the procedure (Wang et al., 2021).
393 Briefly, 15 mg freeze-dried ground root or shoot base tissues were spiked with internal
394 standards D6-ABA (10 ng), D2-GA1 (10 ng), D2-IAA (10 ng), D4-SA (10 ng), and D2-JA
395 (10 ng) along with 750 µL of methanol. The mixture was sonicated for 15 min in an
396 ultrasonic bath (Branson 3510 ultrasonic bath), followed by centrifugation for 5 min at
397 14,000 × g at 4 °C. The supernatant was collected, and the pellet was re-extracted with
398 750 µL of the same solvent. Then, the two supernatants were combined and dried under
399 a vacuum. The sample was re-dissolved in 100 µL of acetonitrile:water (25:75, v-v) and
400 filtered through a 0.22 µm filter for LC-MS analysis.

401
402 Plant hormones were analyzed using LC-MS/MS using UHPLC-Triple-Stage
403 Quadrupole Mass Spectrometer (Thermo Scientific™ Altis™). Chromatographic
404 separation was achieved on the Hypersil GOLD C₁₈ Selectivity HPLC Columns (150 ×
405 4.6 mm; 3 µm; Thermo Scientific™) with mobile phases consisting of water (A) and
406 acetonitrile (B), both containing 0.1% formic acid, and the following linear gradient (flow
407 rate, 0.5 mL/min): 0–10 min, 15%–100 % B, followed by washing with 100 % B for 5 min
408 and equilibration with 15 % B for 2 min. The injection volume was 10 µL, and the column
409 temperature was maintained at 35 °C for each run. The MS parameters of Thermo
410 Scientific™ Altis™ were as follows: positive ion mode for IAA and negative mode for
411 GA, ABA, SA, and JA, ion source of H-ESI, ion spray voltage of 3000 V, sheath gas of
412 40 arbitrary units, aux gas of 15 arbitrary units, sweep gas of 0 arbitrary units, ion
413 transfer tube gas temperature of 350 °C, vaporizer temperature of 350 °C, collision
414 energy of 20 eV, CID gas of 2 mTorr, and full width at half maximum (FWHM) 0.4 Da of
415 Q1/Q3 mass. The characteristic Multiple Reaction Monitoring (MRM) transitions
416 (precursor ion → product ion) were The characteristic MRM transitions (precursor ion →
417 product ion) were 176.2 → 130 for IAA; 263.2 → 153.1, 263.3 → 204.1, 263.3 → 219.1
418 for ABA; 347.2 → 259.1, 347.2 → 273 for GA1; 345.1 → 143, 345.1 → 239 for GA3;
419 137.1 → 93.15, 137.1 → 65.1 for SA; 209.15 → 59.05, 209.15 → 93.04 for JA; 178.2 →
420 132 for D2-IAA; 269.2 → 159.1 for D6-ABA; 349.1 → 261.1 for D2-GA1; 141.0 → 97.0
421 for D4-SA ; 211.0 → 61.0 for D2-JA.

422
423 ***Striga hermonthica* seed germination bioassays**
424 *Striga* seed germination bioassay was carried out based on the protocol (Jamil et al.,
425 2012; Wang et al., 2022). Briefly, 10-day-old pre-conditioning *Striga* seeds were
426 supplied with 50 µL of extracted root exudates of different rice genotypes. After
427 application, *Striga* seeds were incubated at 30 °C in the dark for 24 hours. Germinated
428 (seeds with radicle) and non-germinated seeds were counted under a binocular

429 microscope to calculate germination rate (%) by using SeedQuant software (Braguy et
430 al., 2021).

431

432 **RNA library preparation and transcriptomic analysis**

433 Total rice root RNA was extracted with TRIzol™ (Invitrogen,
434 <https://www.thermofisher.com/de/de/home.html>) using a Direct-zol RNA Miniprep Plus
435 Kit following the manufacturer's instructions (ZYMO RESEARCH; USA). RNA quality
436 was checked with a Agilent 2100 Bioanalyzer, and RNA concentration was measured
437 using a Qubit 3.0 Fluorometer. The cDNA libraries were constructed following standard
438 protocols and paired-end sequenced on an Illumina NextSeq Sequencer (Illumina
439 HiSeq 4000) by Novogene Bioinformatics Technology Co., Ltd. Total reads were
440 mapped to the rice transcripts using HISAT2 (Kim et al., 2019). Differential gene
441 expression was examined using DESeq2 and established by false discovery rate (FDR)
442 ≤ 0.05 (Love et al., 2014).

443

444 **Plant material and growth conditions for *R. irregularis* root colonization**

445 Seed of wild type (cultivar Nipponbare) and independent lines of four *Osmax1* - rice
446 mutants (Os900, Os900/1400, Os1400-12SIII and -12SIV) were germinated in pots
447 containing sand and incubated for ten days in a growth chamber under a 14-h light (23
448 °C)/10-h dark (21 °C). Plants were inoculated with ~1000 sterile spores of *Rhizophagus*
449 *irregularis* DAOM 197198 (Agronutrition, Labège, France). Plants were grown in sterile
450 quartz sand in a growth chamber with the same regime described before and watered
451 with a modified Long-Ashton (LA) solution containing 3.2 µM Na₂HPO₄·12H₂O.

452

453 Mycorrhizal roots were collected at two-time points: 10 days post inoculation (dpi), and
454 40 dpi corresponding to the early and later stages of the mycorrhization process. For
455 the molecular analyses, roots were immediately frozen in liquid nitrogen and stored at
456 -80 °C. At the last time point (40 dpi), mycorrhizal roots were stained with cotton blue
457 (0.1% in lactic acid), and the mycorrhizal colonization level was determined according to
458 Trouvelot et al. (Trouvelot et al., 1986).

459

460 **Transcript analysis of mycorrhizal plants**

461 Total RNA was extracted from rice roots using the Qiagen Plant RNeasy Kit according
462 to the manufacturer's instructions (Qiagen, Hilden; Germany). Following the producer's
463 instructions, samples were treated with TURBO™ DNase (ThermoFischer). The RNA
464 samples were routinely checked for DNA contamination through PCR analysis. Single-
465 strand cDNA was synthesized from 1 µg of total RNA using Super-Script II (Invitrogen),
466 according to the instructions in the user manual. Quantitative RT-PCR (qRT-PCR) was
467 performed using a Rotor-Gene Q 5plex HRM Platform (Qiagen). Each reaction was
468 carried out in a total volume of 15 µL containing 2 µL of diluted cDNA (about 10 ng), 7.5
469 µL of 2× SYBR Green Reaction Mix, and 2.75 µL of each primer (3 µM). The following
470 PCR program was used: 95°C for 90 s, 40 cycles of 95°C for 15 s, and 60°C for 30 s. A
471 melting curve (80 steps with a heating rate of 0.5°C per 10 s and a continuous
472 fluorescence measurement) was recorded at the end of each run to exclude the
473 generation of non-specific PCR products.

474

475 All reactions were performed on at least three biological and two technical replicates.
476 Baseline range and take-off values were automatically calculated using Rotor-Gene Q
477 5plex software.

478

479 The transcript level of *OsPt11* (an AM marker gene) was normalized using the
480 *OsRubQ1* housekeeping gene (Güimil et al., 2005). Only take-off values leading to a
481 mean with a standard deviation below 0.5 were considered. Statistical elaborations
482 were performed using PAST statistical (version 4) (Hammer et al., 2001).

483

484 **Ethynyl deoxyuridine (EdU) staining for cell proliferation analysis**

485 For the EdU staining, the seedlings were transferred to 50 ml falcon tubes containing
486 2 µM 5-ethynyl-2'-deoxyuridine (EdU) in dH₂O, so that the roots were completely
487 submerged in the solution, and kept there for 2 hr. The EdU staining was performed as
488 described previously, using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen,
489 ThermoFisher scientific, USA) (Kirschner et al., 2017). The root were cleared in
490 CLEARSEE clearing solution (Kurihara et al., 2015) at 4 °C in darkness for two weeks,

491 and cell walls were counterstained with 0.1 % Calcofluor White M2R in CLEARSEE
492 overnight in darkness. After washing the roots in CLEARSEE, they were imaged using
493 an inverted confocal microscope (LSM 710, Zeiss) and a 20x objective. Calcofluor
494 White was excited with 405 nm and detected in a detection range of 410 -585 nm. Alexa
495 647 was excited with 633 nm and detected in a detection range of 638 – 755 nm.

496

497 **Statistical analysis**

498 Data are represented as mean and their variations as standard deviation. The statistical
499 significance was determined by one-way analysis of variance (one-way ANOVA) and
500 Tukey's multiple comparison test, using a probability level of $p<0.05$. All statistical
501 elaborations were performed using GraphPad Prism 9.

502

503 **Data availability**

504 All data needed to evaluate the conclusions in the paper are present in the paper and/or
505 the Supplementary Materials. RNA-Seq data can be accessed at NCBI's Gene
506 Expression Omnibus (GEO) via accession number (GSE221837).

507

508 **Author Contributions**

509 S.A.-B. and J.Y.W. proposed the concept. J.Y.W and G.-T. E. C. designed the
510 experiments. J.B. generated transgenic *Osmax1* mutants. J.B. and G.-T. E. C.
511 conducted genotyping. C.V., V.F., and L.L. investigated mycorrhization studies. J.Y.W.
512 and G.-T. E. C. performed LC-MS analysis as well as RNAseq sample preparation and
513 data analysis. G.-T. E. C., G.K.K., and I.B. prepared and performed cellular level
514 analysis. M.J., J.Y.W., and G.-T. E. C. conducted *Striga* bioassays. G.-T. E. C., J.Y.W.,
515 J.B., M.J., L.B., and A.B. conducted phenotyping experiments. G.-T. E. C., J.Y.W., I.B.,
516 L.L., and S. A.-B. analyzed and discussed the data. G.-T. E. C., J.Y.W., J.B., and S. A.-
517 B. wrote the original manuscript. All authors read, edited, and approved the manuscript.

518

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526

527 Competing interests

528 The authors declare no competing interests.

529

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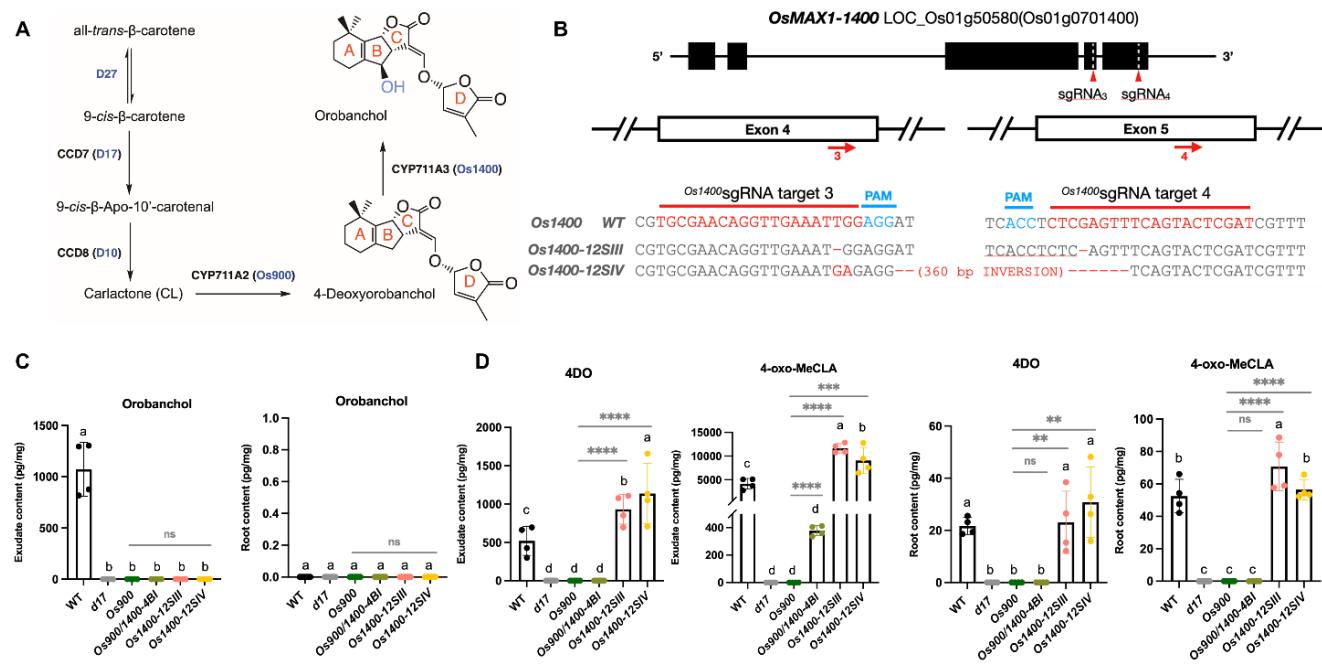
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682 **Figures**



683

684 **Figure 1. Generation of the Os1400-KO lines by CRISPR-Cas9 system. (A)** Scheme
685 of the biosynthesis of the rice canonical SLs (the detailed SL biosynthesis pathway
686 depicted in fig. S1. (B) The structure of the *Os1400* gene and the sequences of the two
687 CRISPR-Cas9 target sites indicated by red arrows 3 and 4. Details of the CRISPR-
688 mediated mutations of the two KO lines, *Os1400-12SIII* and *Os1400-12SIV*, are
689 reported in FigS2. (C) Analysis of SLs in root exudates and root tissues of WT, Os900-
690 *KO line*, Os900/1400-*KO line*, Os1400-*KO lines*, and *d17 mutant* grown under constant
691 low-Pi conditions. The data are presented as means \pm SD for the number of biological
692 replicates n=4 for (C). Significant values determined by one-way ANOVA are shown
693 with different letter (P < 0.05) when compared to WT, and asterisks indicate statistically
694 significant differences as compared to control by two tailed paired Student's t-test (*p <
695 0.05, **p < 0.01; ***p < 0.001; ****p < 0.0001). Abbreviation: 4DO, 4-Deoxyorobanchol

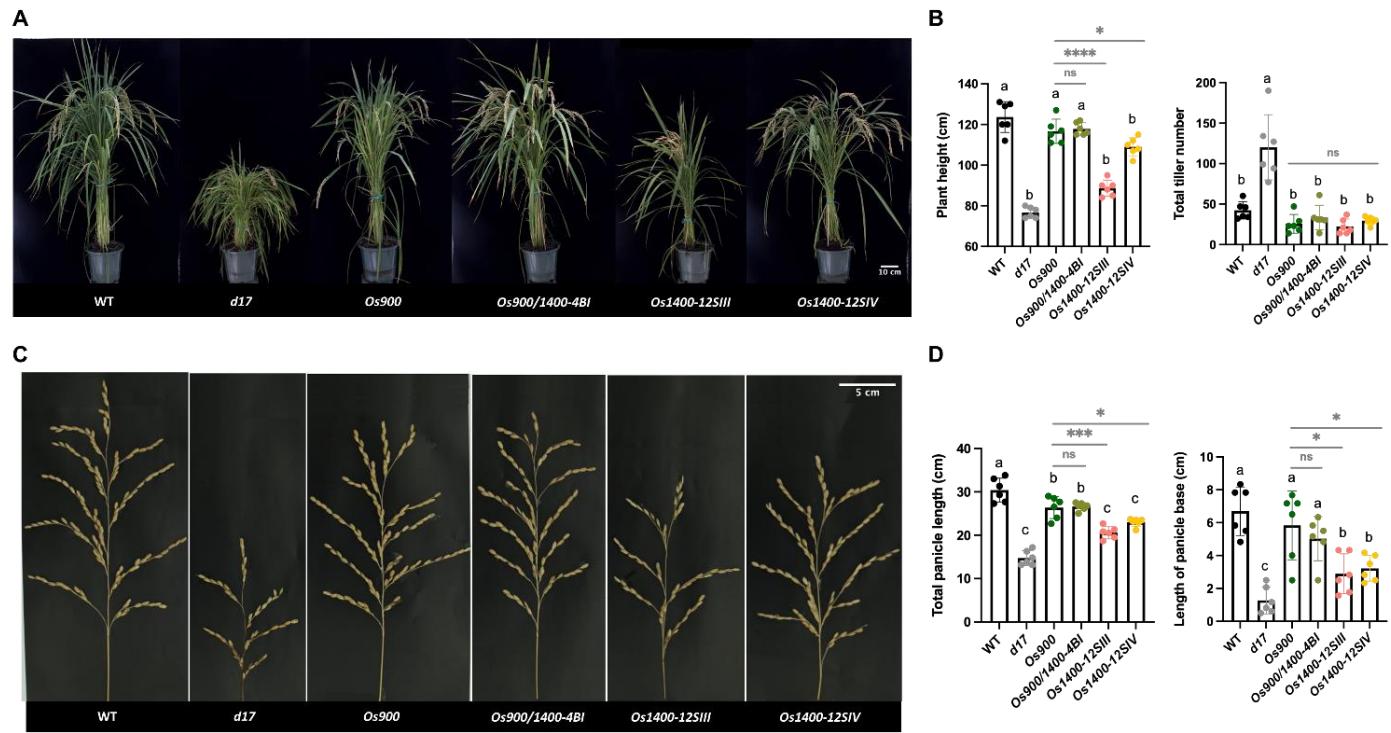
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702 **Figure 2. Phenotypic characterization of *OsMAX1* mutants.** (A and B) Shoot
703 phenotypes of WT, Os900-KO line, Os900/1400-KO line, Os1400-KO lines, and *d17*
704 mutant grown in soil. Scale bar, 10cm. (C and D) Panicle phenotypes of WT, Os900-KO
705 line, Os900/1400-KO line, Os1400-KO lines, and *d17* mutant. Scale bar, 5 cm
706 The data are all presented as means \pm SD for the number of biological replicates n=6.
707 Significant values determined by one-way ANOVA are shown with different letter (P <
708 0.05) when compared to WT, and asterisks indicate statistically significant differences
709 as compared to control by two tailed paired Student's t-test (*p < 0.05, **p < 0.01; ***p <
710 0.001; ****p < 0.0001).

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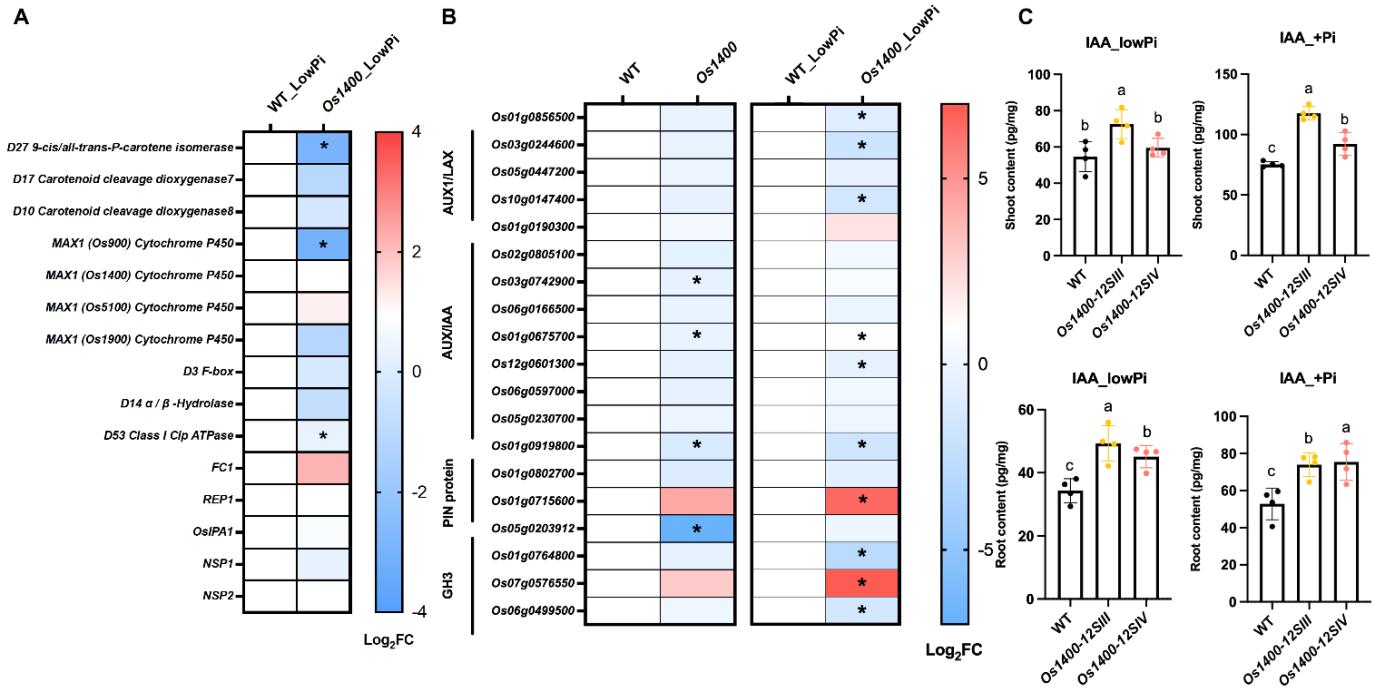
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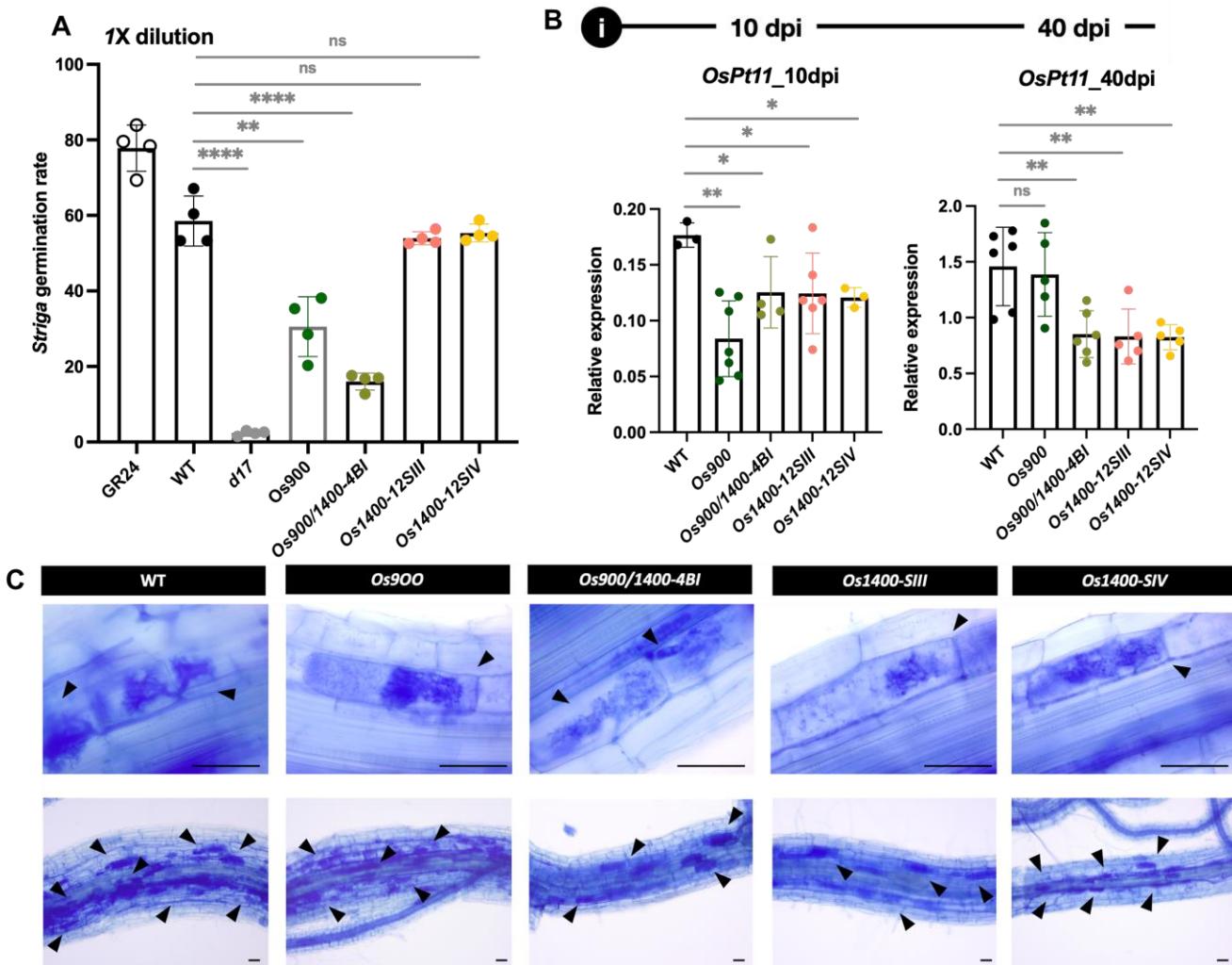


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720 **Figure 3. Transcriptome and hormone analysis of Os1400-KO lines.**

721 (A) Differentially expressed genes (DEGs) of SL biosynthesis and signaling related
 722 gene. Expression pattern was shown in log2FoldChange (Log2FC). Statistically
 723 significant differences indicated by adjusted p- value (*< 0.05). (B) Heat map analysis of
 724 DEGs involved Auxin pathways. AUXIN1/LIKE-AUX1 (AUX1/LAX) are major auxin influx
 725 carriers; AUXIN/indole-3-acetic acid (AUX/IAA) are transcriptional repressors; the PIN-
 726 FORMED (PIN) proteins are secondary transporters in the efflux of auxin; GH3 gene
 727 family encodes auxin-amido synthetases. Expression pattern was shown in
 728 log2FoldChange (Log2FC). Statistically significant differences indicated by adjusted p-
 729 value (*< 0.05). (C) Analysis of IAA (auxin) in root and shoot of WT, and Os1400-KO
 730 lines grown under constant low-Pi and +Pi conditions. Abbreviation: IAA, indole-3-acetic
 731 acid. The data are all presented as means \pm SD for the number of biological replicates
 732 n=3 for (A and B), and n=4 for (C). Significant values determined by one-way ANOVA
 733 are shown with different letter (P < 0.05) when compared to WT, and asterisks indicate
 734 statistically significant differences as compared to control by two tailed paired Student's
 735 t-test (*p < 0.05, **p < 0.01; ***p < 0.001; ****p < 0.0001).

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738 **Figure 4. Assessment of rhizospheric interactions.** Effect of *Os1400*-KO lines on (A)
739 the germination of root parasitic weed *Striga* and (B and C) the arbuscule formation. . .
740 The *R. irregularis* colonization was quantified by measuring the expression of an AM
741 marker gene (*OsPT11*) (B). Arbuscule formation at 10 dpi and 40 dpi. Arrows indicate
742 arbuscule containing cells. Scale bars, 50 μ m (C). The data are all presented as means
743 \pm SD for the number of biological replicates n=4 for (A), and n \geq 3 for (B). Significant
744 values determined by one-way ANOVA are shown with different letter (P < 0.05) when
745 compared to WT, and asterisks indicate statistically significant differences as compared
746 to control by two tailed paired Student's t-test (*p < 0.05, **p < 0.01; ***p < 0.001; ****p
747 < 0.0001).

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