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2 **GTL1 is required for a robust root hair growth response to avoid nutrient**
3 **overloading**

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19 **Keywords:** Arabidopsis thaliana, Excess nutrition, Transcription factor, Root hairs,
20 Gene regulatory network

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22 Summary: 200 words

23 Introduction: 901 words

24 Materials and Methods: 824 words

25 Results: 2924 words

26 Discussion: 1055 words

27

28 **Summary**

- 29 ● Root hair growth is tuned in response to the environment surrounding plants. While
30 most of previous studies focused on the enhancement of root hair growth during
31 nutrient starvation, few studies investigated the root hair response in the presence of
32 excess nutrients.
- 33 ● We report that the post-embryonic growth of wild-type Arabidopsis plants is strongly
34 suppressed with increasing nutrient availability, particularly in the case of root hair
35 growth. We further used gene expression profiling to analyze how excess nutrient
36 availability affects root hair growth, and found that RHD6 subfamily genes, which
37 are positive regulators of root hair growth, are down-regulated in this condition.
- 38 ● On the other hand, defects in GTL1 and DF1, which are negative regulators of root
39 hair growth, cause frail and swollen root hairs to form when excess nutrients are
40 supplied. Additionally, we observed that the RHD6 subfamily genes are
41 mis-expressed in *gtl1-1 df1-1*. Furthermore, overexpression of *RSL4*, an *RHD6*
42 subfamily gene, induces swollen root hairs in the face of a nutrient overload, while
43 mutation of *RSL4* in *gtl1-1 df1-1* restore root hair swelling phenotype.
- 44 ● In conclusion, our data suggest that GTL1 and DF1 prevent unnecessary root hair
45 formation by repressing *RSL4* under excess nutrient conditions.

46

47 **Introduction**

48 Plant biomass accumulation is generally limited by the availability of nutrients,
49 particularly that of the three primary nutrients, nitrogen (N), inorganic phosphate (Pi)
50 and potassium (K). Therefore, fertilizer application is an effective means of increasing
51 plant growth in conditions where nutrients are otherwise lacking (Barker & Pilbeam,
52 2015). Fertilizers supported the past green revolution and have played an essential role
53 increasing the world food supply (Herd et al., 1983). However, because crops are
54 generally not seriously affected by excess nutrients but produce lower yields in the
55 absence of sufficient nutrients, fertilizers are often applied in excess (Barker & Pilbeam,
56 2015). Arabidopsis, the most widely used model plant, also does not typically exhibit
57 obvious growth differences in the presence of excess nutrients, except in the cases of
58 certain heavy metals, B and N sources (Li et al., 2005; Gruber et al., 2013; Esteban et al.,

2016; Yoshinari & Takano, 2017). Thus, plant responses to excess nutrients have been largely overlooked, and the mechanisms plants use to protect themselves from excess nutrition are largely unknown.

Root hairs, which grow from the root epidermis, impact nutrient uptake from the soil, as they increase the surface area of the root system. In *Arabidopsis*, root hairs generally initiate from trichoblasts, one of two types of epidermal cells specified during root development. Atrichoblasts, on the other hand, are root epidermal cells that do not produce hairs under normal conditions (Ishida *et al.*, 2008; Grierson *et al.*, 2014; Salazar-Henao *et al.*, 2016). Root hair initiation, as well as growth of root hairs, is precisely regulated based on nutrient availability. For instance, Pi starvation promotes both root hair formation and growth. Under Pi starvation, root hair number is increased by the initiation of root hairs from atrichoblasts and also by the production of multiple hairs from a single epidermal cell (Ma *et al.*, 2001). At the same time, root hair length increases nearly three-fold in low compared to high phosphate conditions (Bates & Lynch, 1996). Iron deficiency also strongly affects root hair development (Schmidt *et al.*, 2000). In contrast to Pi starvation, however, iron deficiency increases root hair branching rather than promoting ectopic root hair formation (Müller & Schmidt, 2004). These findings suggest that at least some environmental signals affect root hair development independently of each other.

A number of transcription factors have been identified that play key roles regulating root hair development (Ishida *et al.*, 2008; Bruex *et al.*, 2012; Salazar-Henao *et al.*, 2016; Franciosini *et al.*, 2017; Shibata & Sugimoto, 2019; Vissenberg *et al.*, 2020). Atrichoblasts are characterized specifically by the expression of *GLABRA2* (*GL2*), which encodes a transcription factor that functions as a negative regulator of root hair formation and is often used as a marker for non-hair cells (Masucci *et al.*, 1996; Di Cristina *et al.*, 1996; Lin *et al.*, 2015). Conversely, the bHLH transcription factors ROOT HAIR DEFECTIVE6 (*RHD6*) and *RHD6*-LIKE1 (*RSL1*) are key factors that promote hair development (Masucci & Schiefelbein, 1994; Menand *et al.*, 2007; Pires *et al.*, 2013). Following specification of root epidermal cells as trichoblasts, *RHD6* and *RSL1* promote transcription of genes encoding other bHLH transcription factors, including *RSL2*, *RSL4* and *Lotus japonicus* *ROOTHAIRLESS-LIKE 3* (*LRL3*) (Masucci & Schiefelbein, 1994; Karas *et al.*, 2009; Yi *et al.*, 2010). The *rs12 rs14* double mutant

completely lacks root hairs (Yi *et al.*, 2010), indicating that *RSL2* and/or *RSL4* are essential for root hair growth. Similarly, the double or triple mutants for *LRL3* and its homologues *LRL1* and/or *LRL2* have short root hairs that occur at a lower density than normal (Karas *et al.*, 2009; Tam *et al.*, 2015; Breuninger *et al.*, 2016). Thus *LRL3*, together with *LRL1* and *LRL2*, contributes to both root hair formation and growth. In addition to promoting growth of root hairs in response to developmental signals, bHLH transcription factors, particularly *RSL2* and *RSL4*, are also important for root hair growth induced by exogenous phytohormone treatments (auxin, cytokinin, ethylene or jasmonic acid) or nutrient (Pi, N or Fe) deficiency (Yi *et al.*, 2010; Datta *et al.*, 2015; Zhang *et al.*, 2016; Mangano *et al.*, 2017; Feng *et al.*, 2017; Bhosale *et al.*, 2018; Han *et al.*, 2020b). Therefore, *RSL2* and *RSL4* appear to be core factors in the gene regulatory network (GRN) that controls root hair growth (Lee & Cho, 2013; Franciosini *et al.*, 2017; Shibata & Sugimoto, 2019). In addition to these positive regulators of root hair growth, negative regulators have also been identified. The trihelix transcription factor *GT2-LIKE1* (*GTL1*) and its closest homolog, *DF1*, terminate root hair growth by directly repressing *RSL4* together with *RSL4* target-genes (Shibata *et al.*, 2018). In addition, a DOF-type transcription factor, *OBP BINDING PROTEIN 4* (*OBP4*), is a negative regulator of root hair growth, as induction of *OBP4* reduces root hair length (Rymen *et al.*, 2017). Unlike *GTL1* and *DF1*, *OBP4* represses *RSL2* expression and does not affect *RSL4* expression, suggesting that plants have at least two transcriptional pathways that repress root hair growth.

In this study we investigated how root hairs are affected by excess nutrients in *Arabidopsis*. Specifically, we demonstrate that root hair growth is strongly suppressed on double-strength Murashige-Skoog (2xMS) media. Further, we show that the *gtl1-1 dfl-1* mutant forms frail root hairs on 2xMS, suggesting that *GTL1* and *DF1* prevent aberrant root hair development in the presence of excess nutrients. These findings shed light on the mechanisms that plants have evolved to adapt to growth in variable conditions.

Materials and Methods

Plant materials and growth conditions

The *gtl1-1* (WiscDsLox413-416C9), *dfl-1* (SALK_106258), *rsl4-1* (Yi *et al.*, 2010), *rhd6-3* (GABI-Kat 475E09), *obp4-2* (SALK_08510), *obp4-3* (SALKseq_108296),

123 *pGTL1:GTL1-GFP*, *pEXP7:GTL1-GFP*, *pGL2:GL2-GFP/gl2-8*, and *pEXP7:NLS-GFP*
 124 lines were previously described (Breuer *et al.*, 2009, 2012; Yi *et al.*, 2010; Ikeuchi *et al.*,
 125 2015; Rymen *et al.*, 2017; Shibata *et al.*, 2018). *lr13-2* corresponds to SALK_012380 (Fig.
 126 S7). *35S:XVE>>RHD6* (CS2104357, (Coego *et al.*, 2014) were obtained from ABRC. 10
 127 μ M 17 β -estradiol were applied to induce the *RHD6*. Both *RSL4* cDNA and genomic
 128 DNA overexpression lines were generated for this research.

129 Plants for most experiments were grown in a MLR-352H-PJ plant growth
 130 chamber (PHCbi) with a constant temperature of 22°C, at 60% relative humidity and
 131 under continuous light (50-70 μ mol/m²/sec). Half, single or double strength MS media
 132 with 1% sucrose, 0.55 mM myo-inositol, 2.5mM MES-KOH (pH5.7), and 6 g/l Gelzan
 133 (Sigma) were used for plant growth. Each MS medium was made by adding 1 bag of MS
 134 salt mix (Nihon pharmaceutical CO., LTD.) into the proper volume of milli-Q (Merck)
 135 water. For self-made MS media, each chemical solution was prepared and mixed before
 136 autoclaving. FeSO₄ was added together with 2Na-EDTA to avoid precipitation.

137

138 **Plant growth analysis**

139 To measure root hair length, root images were taken from main roots of 12 days old
 140 seedlings with a dissection microscope (Leica M165 FC equipped with a digital Leica
 141 DFC 7000T camera). For the measurements of main root length and lateral root length,
 142 root images from 14 days old seedlings were taken using a scanner (Epson GT-X830).
 143 Subsequently, the shoot tissues from the same seedlings were collected for the
 144 measurements of fresh weight. Five seedlings were pooled and weighed on a
 145 microbalance (METTLER TOLEDO AT200). All image analysis was manually done
 146 with ImageJ (1.53g).

147

148 **Microscopic observation**

149 Expression patterns of *pEXP7:NLS-GFP* and *pGL2:GL2-GFP* were examined using a
 150 Leica SP5 confocal laser scanning microscope with the Leica HyD hybrid detector.
 151 Argon-Ion laser was used for the excitation and the emission light was detected using a
 152 spectral detector. The root images were produced from multiple z-stack images with
 153 maximum projection on LAS-X software (Leica).

154

155 **Plasmid construction and plant transformation**

156 For *35S:RSL4gDNA-GFP* and *35S:RSL4cDNA-GFP*, the coding regions of *RSL4*
157 (AT1G27740.1) were amplified by PCR (PrimeStar max, Takara) using genomic DNA
158 and cDNA as templates, respectively. The PCR products were introduced into
159 pDONR221(Invitrogen) via BP reactions and subsequently transferred to the *pGWB505*
160 destination vector (Nakagawa *et al.*, 2007) via LR reactions. The resulting constructs
161 were then introduced into Arabidopsis plants via the floral dip method (Clough & Bent,
162 1998). A set of primers used for PCR amplification is provided in Table S3.

163

164 **Atomic force microscopy (AFM)**

165 Root sections from 1-week-old seedlings were used for measuring root hair strength. The
166 samples were immobilized by molten 1.5% low-melting point agarose (Agarose LMT
167 1-20K, PrimeGel, Takara Bio Inc.) spread thinly on a glass-bottom dish and immediately
168 covered in water. Intact root hairs were selected for measurement with an inverted
169 microscope (IX71, Olympus). To reduce the effect of leverage, a distance of 30 μm from
170 the root hair base was used as the contact point. Data were obtained using an AFM system
171 (JPK instruments, Nanowizard 4), to which an AFM cantilever (NANOSENSORS,
172 SD-Sphere-NCH, force constant: 42 N/m) was attached. The cantilever tip is shaped into
173 a hemisphere (400 nm radius) to avoid damaging the contact point on the cell. Force
174 curves were observed using contact mode with set point at 130 nN and approach velocity
175 at 2.0 $\mu\text{m/s}$. The data were analyzed by the Hertz model, using JPK Data Processing (ver
176 6.1.158) software, in which the apparent elastic modulus of the cell (stiffness score) was
177 estimated by Young's modulus.

178

179 **RNA extraction and RT-qPCR analysis**

180 Total RNA was extracted from 7-day-old roots using the RNeasy Plant Mini Kit (Qiagen)
181 and QIAcube (Qiagen). Extracted RNA was reverse transcribed using a PrimeScript
182 RT-PCR kit with DNase I (Perfect Real Time) (Takara) in accordance with the
183 accompanying protocol. Transcript levels were determined via qPCR using the
184 THUNDERBIRD SYBR qPCR Mix kit (Toyobo) and Mx399P QPCR system (Agilent).

185 The expression of either the *UBQ10* or *HEL* gene was used as an internal control. A set of
186 primers used for RT-qPCR is provided in Table S3.

187

188 **Promoter-luciferase assay**

189 The effector vectors *35S:GTL1* and *35S:RHD6* were previously described in Shibata et al.,
190 2018 and Rymen et al. 2017, respectively. For the reporter vectors, the promoter regions
191 were amplified by PCR (PrimeSTAR max, Takara) and cloned into pGEM/T-EASY
192 containing LUC+ for *RSL4* and *LRL3*, and R4L1pUGW35 (Nakamura *et al.*, 2009) for
193 *RPS5A* and *RHD6*. Primers used for the cloning are shown in Table S3. As an internal
194 control, the *pPTRL* vector, which drives the expression of a *Renilla LUC* gene under the
195 control of the *CaMV p35S* promoter, was used. The constructs were introduced into
196 Arabidopsis MM2d culture cells (Menges & Murray, 2002) by a gold particle
197 bombardment system. Luciferase activity was quantified using a Mithras LB940
198 microplate luminometer (Berthold Technologies) as described previously (Hiratsu *et al.*,
199 2002).

200

201 **Results**

202 **Excess nutrients inhibit Arabidopsis growth**

203 Murashige-Skoog (MS) medium (Murashige & Skoog, 1962) is widely used for plant
204 growth. For Arabidopsis, half-strength (1/2x) and full-strength (1x) MS media are
205 commonly used as “normal” growth conditions. Thus, in addition to 1/2x and 1x MS
206 media, we prepared double-strength (2x) MS medium as an “excess nutrient” condition to
207 study the impact of excess nutrients on post-embryonic development of Arabidopsis.

208 As shown in Fig.1, plant growth was severely affected by MS strength.
209 Consistent with the visible effect on growth, the fresh weight of 2-weeks-old seedlings
210 (shoot) significantly decreased with increasing MS strength (Fig. 1b). Although the
211 main root length was not significantly altered between the 1/2x and 1xMS conditions,
212 the total lateral root length obviously declined as MS strength increased (Fig. 1d). This
213 observation is consistent with previous reports describing that plants generally reduce
214 lateral root development in the presence of excess nutrients in order to decrease nutrient
215 uptake (Gruber *et al.*, 2013).

216

217 **Double-strength MS medium suppresses root hair growth**

218 In addition to affecting lateral roots, MS concentration also affected root hairs. To study
 219 the impact of excess nutrition on root hair development in greater detail, we observed root
 220 hairs under high magnification (Fig. 2a, left). Consistent with the observation, the root
 221 hair length gradually decreased with increasing MS strength (Fig. 1e). Two weeks after
 222 germination, only extremely short root hairs were observed on 2xMS medium. Close
 223 inspection of root hairs on 2xMS medium revealed that dome-like structures were
 224 established on epidermal cells, indicating that root hair initiation had progressed (Fig. 2b).
 225 To confirm the root hair/non-hair cell identities, we observed the expression patterns of
 226 *EXPANSIN A7 (EXPA7)* and *GL2*, which are hair cell and non-hair cell markers,
 227 respectively (Masucci *et al.*, 1996; Cho & Cosgrove, 2002). In the case of Arabidopsis,
 228 since hair cells and non-hair cells are tandemly arranged within cell files in the root
 229 epidermis, GFP signals from both *pEXP7:NLS-GFP* and *pGL2:GL2-GFP* were observed
 230 in strips (Fig. 2c,d). On 2xMS medium, each GFP signal also aligned within cell files,
 231 indicating that root hair and non-hair cell-pattern was conserved (Fig. 2c,d). In addition,
 232 we also examined the expression levels of *EXPA7* and *GL2* (Fig. S1). For *EXPA7*, the
 233 expression level decreased on 2xMS. This is reasonable because *EXPA7* contributes to
 234 cell expansion (Cho & Cosgrove, 2002), and does not necessarily imply that there are
 235 fewer hair cells in this condition. Importantly, *GL2* expression was also reduced on 2xMS
 236 (Fig. S1). This suggests that the extreme short root hair response is not caused by an
 237 increase of non-hair cells in the root epidermis due to an increase of the *GL2* root hair
 238 identity repressor. Taken together, these results indicate that the root hair phenotype on
 239 2xMS media is caused by defects in cell growth rather than cell specification.

240 We previously identified the trihelix transcription factors *GTL1* and *DF1* as
 241 negative regulators of root hair growth that do not affect root hair cell fate (Shibata *et al.*,
 242 2018). Therefore, we hypothesized that *GTL1* and *DF1* might contribute to the root hair
 243 phenotype on 2xMS medium. To test this hypothesis, we grew the *gtl1-1 dfl-1* mutant on
 244 1/2x, 1x, and 2xMS media and observed the root hair phenotypes (Fig. 2b, right). On
 245 1/2x- and 1xMS media, the *gtl1-1 dfl-1* mutant had longer root hairs compared to those of
 246 the WT, which is consistent with our previous data (Shibata *et al.*, 2018). Strikingly, in
 247 the presence of 2xMS, we found that *gtl1-1 dfl-1* formed tiny root hairs that were more
 248 obvious than the dome-like structures observed in the WT (Fig. 2b). Also, in contrast to

the WT, most root hairs found in *gtl1-1 df1-1* grown on 2xMS were swollen and a few hairs had ruptured (Fig. 2b). These results suggest that GTL1 and DF1 are required for proper termination of root hair growth and/or maintenance of the tube-like structure of root hairs on 2xMS medium.

Swollen root hairs lose stiffness

In order to further investigate the morphological differences between WT and *gtl1-1 df1-1* root hairs, we estimated the stiffness scores of root hairs from analysis of the force-indentation curves acquired by the AFM measurement. First, we confirmed root hair stiffness in plants grown in a normal nutrient condition, i.e. on 1/2xMS medium. In the case of the 1/2xMS condition, no significant difference in the AFM measurement was detected between the WT and *gtl1-1 df1-1* (Fig. 3a,c, S2). The resulting score for the WT was 4.04 ± 3.31 MPa, which was higher than the score obtained from main roots (0.47 ± 0.12 MPa) (Fig. S2b).

On the contrary, for the 2xMS condition, an obvious difference in root hair stiffness was observed in *gtl1-1 df1-1* compared to the WT (Fig. 3b,d). More specifically, the majority of root hairs in WT were stiff, while a few were loose. In contrast, most *gtl1-1 df1-1* root hairs were loose and only a few were stiff (Fig. 3d). Compared to main roots, root hairs in the WT were much stiffer, whereas root hairs and main roots produced similar stiffness readings in *gtl1-1 df1-1* (Fig. S2b). These data indicate that, besides being swollen, the root hairs in *gtl1-1 df1-1* have lost a mechanical property typically associated with this tissue.

A combination of multiple nutrients alters root hair growth

To identify which nutrient(s) cause the strong suppression of root hair growth on 2xMS medium, we prepared custom MS media and altered the ingredients. First, we prepared a medium named “2xMS_All”, which mimics 2xMS made from an MS salt mix (Table S1), and confirmed that root hair growth was strongly inhibited in WT plants grown on this medium (Fig. 4a). Next, to identify which nutrients cause the strong suppression of root hair growth, we prepared various MS media, each in which one ingredient is reduced from the 2xMS level to that of 1/2xMS (Table S2). Among 14 different chemicals found in MS salts, we found that the reduction of NH_4NO_3 alone (2xMS_1/2x NH_4NO_3)

281 partially restored root hair growth (Fig. 4a). Others did not visibly affect root hair growth
282 on 2xMS. Further, to evaluate the impact of N source on root hair growth, we prepared
283 medium with reduced KNO₃ and NH₄NO₃. Although reduction of only KNO₃
284 (2xMS_1/2xKNO₃) did not restore root hair growth, decreased levels of both KNO₃ and
285 NH₄NO₃ (2xMS_1/2xKNO₃ 1/2xNH₄NO₃) further restored root hair growth compared to
286 2xMS_1/2xNH₄NO₃ (Fig. 4a).

287 Additionally, to investigate if double-strength KNO₃ and/or NH₄NO₃ are
288 sufficient to suppress root hair formation, we prepared custom 1/2xMS with extra
289 nutrients (Table S2). First, we confirmed that WT plants form normal root hairs on our
290 self-made 1/2xMS medium (1/2xMS_All, Fig. 4b). Next, we observed root hair
291 phenotypes in plants grown on 1/2xMS media with 2xMS levels of KNO₃
292 (1/2xMS_2xKNO₃), NH₄NO₃ (1/2xMS_2xNH₄NO₃) or both KNO₃ and NH₄NO₃
293 (1/2xMS_2xKNO₃ 2xNH₄NO₃). As opposed to reduction of these nutrients in 2xMS,
294 addition of these nutrients to 1/2xMS did not clearly affect root hair growth. Instead, we
295 found that double-strength iron (1/2xMS_2xFeSO₄+2xEDTA) strongly reduced root
296 hair growth (Fig. 4b), although reduction of the iron source in 2xMS
297 (2xMS_1/2xFeSO₄+1/2xEDTA) had little effect on root hair growth (Fig 4a). The
298 response of root hairs to double-strength iron is consistent with previous studies
299 demonstrating that root hair growth is sensitive to iron levels (Schmidt *et al.*, 2000). It is
300 important to note, however, that double-strength iron or N alone did not reproduce the
301 root hair phenotype observed on 2xMS_All. Therefore, we conclude that the effect of
302 2xMS on root hair growth is likely caused by a combination of multiple nutrients,
303 including N and Fe.

304 In a similar manner, we studied which nutrients cause the root hair swelling in
305 *gtl1-1 df1-1* on 2xMS. First, we confirmed that *gtl1-1 df1-1* forms swollen root hairs on
306 our self-made 2xMS medium (Fig. 4a). Next, we observed root hair morphology of *gtl1-1*
307 *df1-1* on altered 2xMS media, and found that reduction of NH₄NO₃ slightly mitigated the
308 root hair swelling phenotype in *gtl1-1 df1-1* (Fig. 4a). Some of the root hairs of *gtl1-1*
309 *df1-1* grown on 2xMS_1/2xNH₄NO₃ were tube-like rather than balloon-like in structure.
310 We next observed growth of the mutant on 2xMS medium with reduced KNO₃ and
311 NH₄NO₃ (2xMS_1/2xKNO₃ 1/2xNH₄NO₃). As shown in Fig. 4a, the root hair-swelling
312 phenotype in *gtl1-1 df1-1* was further rescued by the reduction of both N sources.

313 However, the majority of root hairs were still partially swollen, which means that the
314 reduction of N alone does not fully restore the root hair phenotype.

315 Furthermore, in order to study if double-strength KNO₃ and/or NH₄NO₃ in 1/2x
316 MS are sufficient to induce root hair swelling in *gtl1-1 df1-1*, we again used our custom
317 MS media series (Table S2). First, we confirmed that *gtl1-1 df1-1* forms normal root hairs
318 on our self-made 1/2xMS (1/2xMS_All, Fig 4a), and then observed root hair phenotypes
319 on the 1/2xMS media containing extra nutrients. Consistent with both Johnson and
320 commercial MS media, *gtl1-1 df1-1* had longer root hairs on self-made 1/2xMS compared
321 to the WT. On the contrary, root hair formation of *gtl1-1 df1-1* was strongly inhibited by
322 addition of NH₄NO₃ to 1/2xMS (1/2xMS_2xNH₄NO₃). Moreover, addition of KNO₃ to
323 1/2xMS with double-strength 2x NH₄NO₃ (1/2xMS_2xKNO₃ 2xNH₄NO₃) further
324 inhibited root hair formation. In addition, double-strength iron
325 (1/2xMS_2xFeSO₄+2xEDTA) completely suppressed root hair growth in *gtl1-1 df1-1*
326 (Fig. 4b). Notably, such a severe phenotype was only observed in *gtl1-1 df1-1* grown on
327 1/2xMS_2xFeSO₄+2xEDTA and not in the WT. These results suggest that *gtl1-1 df1-1*
328 is hypersensitive to environmental effects on root hair growth. However, from these
329 experiments, we were unable to identify the specific ingredients in 2xMS that trigger root
330 hair swelling. This likely indicates that a combination of multiple nutrients causes root
331 hair swelling in *gtl1-1 df1-1*.

332

333 ***RHD6*, *RSL4* and *LRL3* are differentially expressed in *gtl1-1 df1-1***

334 Based on the root hair phenotypes, we concluded that WT plants suppress root hair
335 growth on 2xMS via the activity of GTL1 and DF1. Thus, we hypothesized that *GTL1*
336 and *DF1* are induced by higher MS concentrations. In order to test this hypothesis, we
337 measured mRNA levels of *GTL1* and *DF1* by RT-qPCR. However, contrary to
338 expectations, the expression of both *GTL1* and *DF1* actually declined on 2xMS
339 compared to 1/2xMS (Fig. S3), suggesting that activity of GTL1 and DF1 is regulated
340 by non-transcriptional mechanisms.

341 We next investigated the molecular mechanisms by which the WT suppresses
342 root hair growth on 2xMS and how defects in *GTL1* and *DF1* cause root hair swelling on
343 2xMS. Since members of the RHD6 subfamily generally play key roles promoting root
344 hair growth in response to environmental signals (Franciosini *et al.*, 2017; Shibata &

345 Sugimoto, 2019), we investigated if the expression of the genes encoding these
346 transcription factors is affected by MS strength. Between the WT and *gtl1-1 df1-1*, we
347 found that *RHD6*, *RSL2*, *RSL3*, *RSL4* and *LRL3* were differentially expressed in at least
348 one condition (Fig. 5). Among these genes, *RHD6*, *RSL4* and *LRL3* were expressed at
349 higher levels in *gtl1-1 df1-1*, which is consistent with our previous data (Shibata *et al.*,
350 2018). Interestingly, the expression of *RHD6* and *RSL4* was reduced on 2xMS compared
351 to both 1/2xMS and 1xMS. Additionally, the expression levels of these genes in *gtl1-1*
352 *df1-1* on 2xMS were similar to that in the WT on 1/2xMS or 1xMS, suggesting that the
353 suppression of *RHD6* and *RSL4* expression on 2xMS is deficient in *gtl1-1 df1-1*. In
354 contrast to *RHD6* and *RSL4*, *RSL2* and *RSL3* levels were lower in *gtl1-1 df1-1* compared
355 to the WT in at least one condition. Notably, the expression of both *RSL2* and *RSL3* was
356 significantly reduced in *gtl1-1 df1-1* on 2xMS. These data indicate that *gtl1-1 df1-1*
357 causes stronger suppression of *RSL2* and *RSL3* expression on 2xMS.

358 In addition to *RHD6* and *RSL4* mentioned above, the levels of *RSL2*, *RSL3*, and
359 *LRL3* declined on 2xMS compared to other media in the WT (Fig. 5). These expression
360 profiles are consistent with the near lack of root hair growth in the WT on 2xMS. The
361 *gtl1-1 df1-1* mutant also exhibited reduced expression of these *RHD6* subfamily genes on
362 2xMS, demonstrating that other suppression mechanisms are still active. A Dof-type
363 transcription factor OBP4 was characterized as a suppressor of *RSL2* and *RSL3* that
364 inhibits root hair growth in response ABA signaling (Rymen *et al.*, 2017). To study
365 whether OBP4 is required for the repression of *RSL2* and *RSL3* on 2xMS, we analyzed
366 *obp4-2* (SALK_085101, knock-down allele) and *obp4-3* (SALKseq_108296, knock-out
367 allele) mutants across the MS dilution series. The roots of *obp4-2* and *obp4-3* resembled
368 the WT on 2xMS, not forming swollen root hairs as potentially might be expected if the
369 expression of *RSL2* and *RSL3* was elevated in this mutant (Fig. S4). More importantly,
370 the expression levels of *RSL2* and *RSL3* on 2xMS were unaffected in both *obp4* mutants
371 (Fig. S5). Collectively, these results indicate that the decline of *RSL2* and *RSL3*
372 expression on 2xMS is independent from GTL1, DF1 and OBP4.

373

374 **GTL1 suppresses *RHD6*, *RSL4* and *LRL3***

375 Based on our gene expression analysis, *RHD6*, *RSL4* and *LRL3* levels were elevated in
376 *gtl1-1 df1-1* on at least one of the three types of MS media (Fig. 5). Thus, we

hypothesized that *RSL4*, *RHD6* and *LRL3* are regulated by GTL1. In order to test this hypothesis, we used a promoter-luciferase assay employing MM2d, an Arabidopsis culture cell line. Using this approach, we found that *GTL1* overexpression strongly reduced *RHD6*, *RSL4* and *LRL3* promoter activities compared to the control (empty) vector (Fig. 6a,b), demonstrating that *RHD6*, *RSL4* and *LRL3* are under the control of GTL1.

Next, since *RSL4* is known as a direct target of RHD6 and GTL1 (Yi *et al.*, 2010; Shibata *et al.*, 2018), we studied the relationship between GTL1 and RHD6 on *RSL4* promoter activity. As shown in Fig. 7(a), we reproduced that RHD6 and GTL1 work as an activator and a repressor of *RSL4* promoter activity, respectively, in culture cells, consistent with their effects on *RSL4* expression *in planta* (Yi *et al.*, 2010; Shibata *et al.*, 2018). When both of the transcription factors are introduced together into culture cells, we found that GTL1 dominantly represses *RSL4* promoter activity (Fig. 7a). To identify the regions within the *RSL4* promoter important for GTL1- and RHD6-mediated regulation, a truncated series of *RSL4* promoters were used in another promoter-luciferase assay. When we used a 1500bp region directly upstream from the start codon of *RSL4*, both RHD6 and GTL1 altered *pRSL4:LUC* activity (Fig. 7b,c). However, when the fragment was shortened to contain only 500bp immediately upstream of the *RSL4* start codon, RHD6 no longer activated *pRSL4:LUC*, while GTL1 still repressed expression of the reporter (Fig. 7b,c). These results indicate that RHD6 and GTL1 bind different regions of the *RSL4* promoter and regulate its expression independently.

Ectopic expression of *RSL4* induces abnormal root hairs

Given that *RHD6*, *RSL4*, and *LRL3* promote root hair growth and are repressed by GTL1/DF1 *in planta*, mis-expression of these genes in *gtl1-1 df1-1* may cause the root hair swelling observed in this mutant on 2xMS. In order to test this hypothesis, we generated *rhb6 gtl1 df1*, *rs14 gtl1 df1* and *lrl3 gtl1 df1* triple mutants. Then, root hair phenotypes were examined on 2xMS.

For *RHD6*, *rhb6-3* is almost hairless even under normal conditions (Masucci & Schiefelbein, 1994; Menand *et al.*, 2007), while the *rhb6-3 gtl1-1 df1-1* mutant formed tiny root hairs with balloon-like structures on 2xMS (Fig. 8a), suggesting that RHD6 is

not responsible for the phenotype observed in *gtl1-1 df1-1*. In order to further investigate if ectopic expression of *RHD6* causes root hair swelling on 2xMS, we used an *RHD6*-inducible overexpression line (*35S:XVE>>RHD6*), in which 10 μ M 17 β -estradiol (17ED) treatment increased the level of *RHD6* mRNA by approximately 180-fold compared to mock treatment with DMSO (Fig. S6). Consistent with the WT phenotype, *35S:XVE>>RHD6* treated with DMSO did not form root hairs on 2xMS (Fig. 8b). On the other hand, *35S:XVE>>RHD6* treated with 17ED on 2xMS produced root hairs with normal morphology, in contrast to the expectation that it might cause swollen hairs to form (Fig. 8b). These results demonstrated that the mis-expression of *RHD6* in *gtl1-1 df1-1* does not cause the swollen-root hair phenotype on 2xMS, but instead that *RHD6* promotes the formation of proper root hairs on 2xMS.

For *LRL3*, the *lrl3-2* single mutant displayed a similar suppression of root hair growth on 2xMS compared to the WT (Fig. 8a, S7). This is consistent with a previous finding that another single mutant for *LRL3*, *lrl3-1*, does not affect root hair growth (Karas *et al.*, 2009). Similar to *gtl1-1 df1-1*, *lrl3-2 gtl1-1 df1-1* formed swollen root hairs on 2xMS (Fig. 8a). Therefore, mis-expression of *LRL3* in *gtl1-1 df1-1* does not cause the formation of abnormal root hairs in *gtl1-1 df1-1* on double-strength MS.

In contrast to *RHD6* and *LRL3*, we found that mutation of *RSL4* rescued the *gtl1-1 df1-1* root hair phenotype on 2xMS. (Fig. 8a). Although the *rsl4* single mutant forms root hairs under normal condition due to redundancy with *RSL2* (Shibata *et al.*, 2018), both *rsl4-1* and *rsl4-1 gtl1-1 df1-1* exhibited almost no root hair growth on 2xMS. To further investigate if ectopic expression of *RSL4* leads to root hair swelling, we produced lines constitutively overexpressing *RSL4*, using the 35S promoter. These lines exhibited root hair swelling on 2xMS, indicating that mis-expression of *RSL4* causes this phenotype (Fig. 8c). Taken together with the phenotype of the *rsl4-1 gtl1-1 df1-1* triple mutant, these data strongly suggest that mis-expression of *RSL4* in *gtl1-1 df1-1* causes abnormal root hair formation on double-strength MS. Notably, among the *RHD6* subfamily genes, *RSL4* was the only one which we observed to be overexpressed in *gtl1-1 df1-1* on 2xMS (Fig. 5).

438

439 Discussion

440 Environmental signals affect post-embryonic development of plants. In this study, we
 441 showed that excess nutrients, supplied in the form of double-strength MS media, strongly
 442 inhibit Arabidopsis growth and have a particularly striking effect on root hairs (Fig. 1).
 443 Since one of the main functions of root hairs is to enhance nutrient uptake, the root hair
 444 response to 2xMS likely reduces nutrient overloading. Our data further indicate that
 445 individual MS components do not account for the effect of 2xMS on root hair growth.
 446 Specifically, our custom MS experiments showed that neither the reduction of individual
 447 nutrients in 2xMS or their increase in 1/2xMS completely reproduces the root hair
 448 phenotypes observed on 1/2xMS or 2xMS, respectively (Fig. 4). These data suggest that
 449 Arabidopsis plants integrates signals transduced in response to multiple nutrients and
 450 determines an appropriate root hair response.

451

452 **GTL1 and DF1 are required for proper root hair response to excess nutrients**

453 We demonstrated that GTL1 and DF1 are critical for suppression of root hair growth on
 454 2xMS, with the *gtl1-1 df1-1* mutant forming frail root hairs on 2xMS (Fig. 2-3). Our AFM
 455 analysis showed that both WT and *gtl1-1 df1-1* reduce stiffness of root hairs. However,
 456 the reduction of root hair stiffness was more pronounced in *gtl1-1 df1-1* than in the WT
 457 (Fig. 3). Consistent with this result, root hair growth of *gtl1-1 df1-1* is hypersensitive to
 458 reduction/addition of nutrients (Fig. 4). Additionally, in the *gtl1-1 df1-1* mutant, the
 459 expression of *RHD6* subfamily genes was not properly regulated, with some genes, like
 460 *RSL4*, up-regulated in this mutant on 2xMS, while others, namely *RSL2* and *RSL3*, were
 461 down-regulated (Fig. 5). Thus, we conclude that GTL1 and DF1 function to stabilize
 462 changes in gene expression induced by variable environments, thus ensuring appropriate
 463 root hair responses.

464 Notably, the expression levels of *GTL1* and *DF1* gradually decrease with
 465 increasing MS concentration, indicating that the strong suppression of root hair growth on
 466 2xMS is not due to induction of *GTL1* and *DF1* (Fig. S3). Instead, nutrient signaling
 467 might affect the activities of GTL1 and DF1 via post-translational modifications. Some
 468 publicly available proteomic data sets indicate that GTL1 undergoes post-translational
 469 modifications, including phosphorylation and SUMOylation (Reiland *et al.*, 2009, 2011;
 470 Umezawa *et al.*, 2013; Wang *et al.*, 2013; Roitinger *et al.*, 2015; Choudhary *et al.*, 2015).

471 In another study, it was reported that a MAP kinase, MPK4, binds GTL1, although
472 phosphorylation of GTL1 by MPK4 was not detected (Völz *et al.*, 2018). In addition,
473 GTL1 activity is known to be regulated by Ca^{2+} -dependent calmodulin ($\text{Ca}^{2+}/\text{CaM}$).
474 $\text{Ca}^{2+}/\text{CaM}$ binds the DNA binding domain of GTL1 and inhibits DNA binding (Weng *et al.*,
475 2012; Yoo *et al.*, 2019). Interestingly, it has also been suggested that GTL1
476 transcriptional-repressor activity is inhibited by $\text{Ca}^{2+}/\text{CaM}$ in response to water-deficit
477 stress, thus leading to derepression of genes related to stomata development (Yoo *et al.*,
478 2010, 2019; Weng *et al.*, 2012). These data, together with the lack of transcriptional
479 activation of *GTL1* and *DF1* on 2xMS compared to 1/2xMS, suggest the activity of GTL1
480 and DF1 is likely regulated by post-translational modification(s) in the context of excess
481 nutrients.

482

483 **A GRN enables precise regulation of genes implicated in root hair growth**

484 We demonstrated that WT Arabidopsis plants strongly suppress root hair growth on
485 2xMS by repressing *RHD6* subfamily genes. Among these genes, at least *RHD6*, *RSL4*
486 and *LRL3* are under the control of GTL1, while the suppression of *RSL2* and *RSL3*
487 appears to be independent of GTL1 and DF1. However, even *RHD6*, *RSL4* and *LRL3*
488 were still responsive to changes in MS concentration in *gtl1-1 df1-1*, suggesting that other
489 transcription factors besides GTL1 and DF1 contribute to repression of these genes (Fig.
490 5). A C2H2 transcription factor, ZINC-FINGER-PROTEIN1 (ZP1), was recently shown
491 to suppress root hair growth by directly repressing *RHD6*, *RSL2* and *RSL4* (Han *et al.*,
492 2020a). Although this study did not investigate if ZP1 works in the context of
493 environmental responses, it is possible that ZP1 contributes to the repression of root hair
494 growth on 2xMS together with GTL1 and DF1. MYB DOMAIN PROTEIN 30 (MYB30)
495 was also shown recently to negatively regulate root hair growth via direct repression of
496 *RSL4* (Xiao *et al.*, 2021). Notably, MYB30 inhibits ETHYLENE INSENSITIVE 3
497 (EIN3), a positive regulator of ethylene signaling, by directly interacting with this protein
498 (Xiao *et al.*, 2021). EIN3, on the other hand, is known to bind *RHD6* and promotes root
499 hair growth in response to ethylene signaling (Song *et al.*, 2016b). Thus, MYB30 is
500 expected to regulate *RSL4* competitively with EIN3. Additionally,
501 JASMONATE-ZIM-DOMAIN PROTEIN (JAZ) proteins, which are key regulators of
502 jasmonic acid (JA) signaling, also physically interact with *RHD6*, thus affecting root hair

development in response to JA (Han *et al.*, 2020b). These data suggest that RHD6 and its binding partners work as a hub within the GRN that regulates root hair development in response to environmental signals. Moreover, our data showed that GTL1 works oppositely to RHD6 in the regulation of *RSL4*. *RSL4* itself is also known as a master regulator of root hair growth, promoting growth by directly activating root hair-specific genes (Hwang *et al.*, 2017). Interestingly, *RSL4* also forms a feedback loop with GTL1 (Shibata *et al.*, 2018). Induction of *RSL4* activity in glucocorticoid receptor:*RSL4* (GR:*RSL4*)-expressing plants showed that *RSL4* induces *GTL1* expression (Vijayakumar *et al.*, 2016). On the other hand, we previously demonstrated that GTL1 directly suppresses *RSL4* expression (Shibata *et al.*, 2018), similar to the negative effect of GTL1 on *RSL4* observed in this study (Figs. 5-7). Evaluation of the biological significance of this feed-back-loop between GTL1 and RHD6 requires further analysis, using approaches such as mathematical modeling.

Regarding the relationship between *RHD6* and *RSL4*, we showed that the ectopic overexpression of *RHD6* causes plants to form normal root hairs on 2xMS, while *RSL4* does not have this effect (Fig. 8b,c). These results indicate that RHD6 and *RSL4* promote the formation of robust and frail root hairs, respectively, on 2xMS. As RHD6 works upstream of *RSL4*, our data suggest that coordinated induction of other TFs by RHD6 in addition to *RSL4*, such as *RSL2*, is important for proper root hair growth in this condition. Since in general plant responses to environmental signals are tightly controlled by a GRN consisting of multiple TFs (Song *et al.*, 2016a; Van den Broeck *et al.*, 2020), the regulatory network revealed in this study likely fine tunes root hair growth in response to fluctuating environmental conditions.

526

527 Accession numbers

<i>GTL1</i>	AT1G33240	<i>gtl1-1</i>	WiscDsLox413-416C9
<i>DF1</i>	AT1G76880	<i>df1-1</i>	SALK_106258
<i>RSL4</i>	AT1G27740	<i>rsl4-1</i>	GT_5_105706 (Yi <i>et al.</i> , 2010)
<i>RHD6</i>	AT1G66470	<i>rh6-3</i>	GABI-Kat 475E09
		<i>35S:XVE>>RHD6</i>	CS2104358

<i>OBP4</i>	AT5G60850	<i>obp4-2</i>	SALK_085101
<i>OBP4</i>	AT5G60850	<i>obp4-3</i>	SALKseq_108296
<i>GL2</i>	AT1G79840	<i>gl2-8</i>	CS2106731

528

529 **Acknowledgments**

530 We are grateful to the members of the Sugimoto lab for fruitful discussions about the
531 manuscript. We thank Akiko Hanada, Mariko Mouri, Chika Ikeda and Noriko Doi for
532 their technical assistance. We thank Kazunori Okano and Eri Akita for their technical
533 support with the AFM analysis. We thank Dr. June-Sik Kim for advice regarding
534 statistical tests. This work was supported by grants from MEXT KAKENHI Grant-in-Aid
535 for JSPS Fellows (16J07464) and Grant-in-Aid for Young Scientists (20K15827) to M.S.,
536 Grant-in-Aid for JSPS Fellows (19F19781) to D.S.F., Grant-in-Aid for Scientific
537 Research on Priority Areas “Plant-Structure Optimization Strategy” (JP18H05493) to
538 Y.H. and Grant-in-Aid for Transformative Research Areas (A) “Plant Resilience under
539 Fluctuating Environment” (20H05911) to K.S.

540

541 **Author contribution**

542 M.S., D.S.F., B.R. and K.S. conceived the research and designed the experiments. M.S.,
543 and A.K. performed most of the experiments. R.T., and Y.H. performed AFM analysis.
544 M.S., D.S.F., Y.H. and K.S. wrote the manuscript with input from all co-authors.

545

546

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743

744

745 **Figure Legends**

746 **Fig. 1. Arabidopsis growth is repressed by higher concentrations of MS.**

747 (a) Images of 3-week-old WT plants grown on half- (1/2x), full- (1x) or double- (2x)
748 strength MS medium. Scale bar = 1 cm. Quantitative analysis of fresh weight (b), main
749 root length (c), sum of lateral root (LR) length (d) and length of 20 longest root hairs from
750 each seedling (e). Data are mean $\pm$ SE (n = 40 for (b), 16 for (c) and d, 12 for (e)).
751 Different letters indicate significant differences One-way ANOVA with post-hoc Tukey
752 HSD test, $p < 0.01$).

753

754 **Fig. 2. GTL1/DF1 prevent formation of aberrant root hairs on 2xMS by repressing** 755 **root hair growth.**

756 (a) Images of root tips of WT and *gtl1-1 df1-1* on half- (1/2x), full- (1x) or double- (2x)
757 strength MS medium. High-magnification images of roots grown on 2xMS media are
758 shown in (b). The arrow head in (b) shows a root hair initiation site from an epidermal cell.
759 Scale bars = 1000 μ m in (a) and 100 μ m in (b). (c, d) Confocal microscope images of
760 *pEXP7:NLS-GFP* (c) and *pGL2:GL2GFP/gl2-8 (pGL2:GL2-GFP)* (d) on 1/2xMS and
761 2xMS. *EXP7* and *GL2* are used as hair cell and non-hair cell markers, respectively. Scale
762 bars = 200 μ m in (d) and 100 μ m in (e).

763

764 **Fig. 3. GTL1/DF1 maintain root hair stiffness on 2xMS.**

765 (a,b) Microscopic images of root hairs used for AFM analysis. The cantilever indicates
766 the root hairs used for measurement. Scale bars = 100 μ m in (a) and 30 μ m in (b).
767 (c) Box plots showing root hair stiffness scores, as determined from AFM analysis. Y axis
768 indicates Young's modulus (*E*). Asterisks indicate a significant difference (Student's
769 t-test, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$).

770

771 **Fig. 4. Multiple nutrients contained in MS media affect root hair growth.**

772 Images of root hairs grown on custom MS media. 2xMS- and 1/2xMS-based medium
773 are shown in (a) and (b), respectively. Upper and lower panels show root hairs of WT
774 and *gtl1-1 df1-1* in each condition, respectively. See Table S2 for a complete list of
775 ingredients. Scale bars = 200 μ m.

776

777 **Fig. 5. The expression of root hair regulators is affected by MS concentration.**

778 RT-qPCR analysis of key transcription factors regulating root hair growth in WT and
779 *gtl1-1 df1-1* grown on 1/2x, 1x and 2x MS media. Expression levels are normalized to
780 that of the *HEL* gene. Data are mean $\pm$ SD. (n = 3, biological replicates). Asterisks
781 indicate a significant difference compared to the WT grown on the same type of medium
782 (Student's t-test, *p < 0.05, **p < 0.01). Different letters indicate significant differences
783 between media conditions within the same genotype (One-way ANOVA with post-hoc
784 Tukey HSD test, p < 0.05).

785

786 **Fig. 6. GTL1 suppresses *RHD6*, *RSL4* and *LRL3* expression**

787 (a) Constructs used for the promoter-Luciferase assay.

788 (b) Results from the promoter-Luciferase assay using MM2d culture cells. GTL1 was
789 used as the effector. *RPS5A*, *RHD6*, *RSL1*, or *RSL4* promoter sequences fused with
790 Luciferase were used as reporters. *RPS5A* served as the negative control. Data are mean
791 $\pm$ SD (n = 3). Asterisks indicate significant difference compared to vector control
792 (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001)

793

794 **Fig. 7. GTL1 dominantly represses *RSL4***

795 (a) *RHD6* and *GTL1* were both used as effectors in an MM2d-based promoter-Luciferase
796 assay. The *RSL4* promoter region (1500 bp upstream from the start codon) fused with
797 Luciferase was used as a reporter. Different letters indicate significant differences
798 (One-way ANOVA with post-hoc Tukey HSD test, p < 0.01) compared to the control
799 vector (empty).

800 (b,c) Promoter-Luciferase assay using a series of truncated *RSL4* promoter fragments.
801 GTL1 and RHD6 were used as effectors in (b) and (c), respectively. Data are mean $\pm$ SD
802 (n = 3). Asterisks indicate significant differences compared to vector control (Student's
803 t-test, *p < 0.05, **p < 0.01, ***p < 0.001)

804

805 **Fig. 8. *RSL4* causes aberrant root hair formation on 2xMS**

806 (a) Root hairs of *rhhd6-3*, *lrl3-2*, *rsl4-1* mutants in the WT (upper panel) or *gtl1-1 df1-1*
807 (lower panel) background grown on 2xMS medium. Note that only the *rsl4* mutation
808 mitigated the root hair swelling phenotype of *gtl1-1 df1-1*.

809 (b) Formation of normal root hairs on 2xMS in plants ectopically over-expressing *RHD6*.
 810 DMSO was used as a control treatment. See also Fig. S6.
 811 (c) Swollen root hairs in plant ectopically over-expressing *RSL4* on 2xMS.
 812 Overexpression of constructs derived from either cDNA (*35S:RSL4cds-GFP*) or the
 813 genomic sequence (*35S:gRSL4-GFP*) of *RSL4* produced similar phenotypes.
 814 Scale bars = 200 μ m.

815

Supplemental Data

Fig. S1. The expression levels of *EXPA7* and *GL2*

818 RT-qPCR analysis of *EXPA7* and *GL2* in WT and *gtl1-1 df1-1* grown on 1/2x, 1x and 2x
 819 MS media. Expression levels are normalized to that of the *HEL* gene. Data are mean +/-
 820 SD. (n = 3, biological replicates). Asterisks indicate a significant difference for the same
 821 genotype grown on different types of media (Student's t-test, *p < 0.05, **p < 0.01, ***p
 822 < 0.001).

823

Fig. S2. Measurement of root and root hair stiffness

825 (a) Force-indentation curves in the AFM measurement.
 826 (b) Box plots showing main root stiffness scores, as determined from analysis of the force
 827 indentation curves. Y axis indicates Young's modulus (*E*). Asterisks indicate a significant
 828 difference (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001).

829

Fig. S3. The expression levels of *GTL1* and *DF1*

831 RT-qPCR analysis of *GTL1* and *DF1* grown on 1/2x, 1x and 2x MS media. Expression
 832 levels are normalized to that of the *HEL* gene. Data are mean +/- SD. (n = 3, biological
 833 replicates). Different letters indicate significant differences for the same genotype grown
 834 on different types of media (One-way ANOVA with post-hoc Tukey HSD test, p < 0.05).

835

Fig. S4. Roots of *obp4* mutants grown on MS dilution series

837 Images of root tips of the WT, *obp4-2* and *obp4-3*, on half- (1/2x), full- (1x) or double-
 838 (2x) strength MS medium. Scale bars = 1000 μ m

839

Fig. S5. The expression levels of *RSL2* and *RSL3* in *obp4* mutants

840

RT-qPCR analysis of *RSL2* and *RSL3* grown on 1/2x, 1x and 2x MS media. Expression levels are normalized to that of the *HEL* gene. Data are mean $\pm$ SD. (n = 3, biological replicates). Asterisks indicate a significant difference compared to the WT grown on the same type of medium (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. S6. The expression levels of *RHD6* and *RSL4* in corresponding overexpression lines

(a) RT-qPCR analysis of *RHD6* in *35S:XVE>>RHD6*. Total RNA was purified after 24h of treatment with 10 μ M 17ED. DMSO was used as a control treatment. Expression levels are normalized to that of the *UBQ10* gene. Data are mean $\pm$ SD. (n = 3, biological replicates). Asterisks indicate a significant difference compared to the control condition (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001).

(b) RT-qPCR analysis of *RSL4* in *35S:RSL4cds #1-2* and *35S:gRSL4 #2-2*. Expression levels are normalized to that of the *UBQ10* gene. Data are mean $\pm$ SD. (n = 3, biological replicates). Asterisks indicate a significant difference compared to the WT grown on the same type of medium (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. S7. Properties of the *LRL3* knock-down mutant

(a) Gene structure of *AT5G58010/LRL3*. Closed boxes denote exons. The triangle represents the position of the T-DNA insertion (SALK_012380/*lr3-2*). Arrows indicate primers used for genotyping.

(b) Image of an agarose gel showing PCR-amplified DNA fragments for genotyping. Primer sets are shown above each lane. L indicates 100bp Ladder.

(c) Relative expression level of *LRL3* in the *lr3-2* mutant.

(d) Nucleotide sequence of the genomic region containing the *LRL3* gene. The region between start codons of *LRL3* and *AT5G58020* was used as the *LRL3* promoter in this study.

The sequence and splicing data were obtained from the TAIR database (<http://www.arabidopsis.org>).

Table S1. Components of 1/2x, 1x and 2x MS media

Table S2. Components of the custom MS media

873 **Table S3. List of PCR Primers used in this study**

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