1   **Title**

2   Root-specific reduction of cytokinin perception enhances shoot growth with an alteration of *trans*-zeatin

3   distribution in *Arabidopsis thaliana*

4   **Running head**

5   Root-specific reduction of cytokinin perception

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22 **Abstract**

23 Compelling evidence demonstrates that root-derived cytokinins (CKs) contribute to shoot growth via

24 long-distance transport; therefore, we hypothesized that an increase in root-derived CKs enhances shoot

25 growth. To demonstrate this, we grafted Arabidopsis Col-0 (WT) scion onto rootstock originated from WT

26 or a double-knockout line of CK receptors *AHK2* and *AHK3* (*ahk23*) because the knockout line over

27 accumulates CKs in the body due to feedback homeostasis regulation. The grafted plants (scion/rootstock:

28 WT/WT and WT/*ahk23*) were grown in vermiculite pots or solid media under high and low nitrogen

29 regimes for vegetative growth and biochemical analysis. The root-specific deficiency of *AHK2* and *AHK3*

30 increased root concentrations of *trans*-zeatin (tZ)-type and N^6-(Δ^2-isopentenyl) adenine (iP)-type CKs,

31 induced CK biosynthesis genes, and repressed CK degradation genes in the root. Shoot growth, shoot

32 concentrations of tZ-type CKs, and shoot expression of CK-inducible marker genes were consistently

33 larger in the WT/*ahk23* plants than in the WT/WT plants. Moreover, the root-specific deficiency of *AHK2*

34 and *AHK3* enhanced shoot growth in the WT scion more strongly than in the *ahk23* scion. Given that

35 tZ-type CKs are predominantly produced from iP-type CKs in the root and xylem-mobile, it is concluded

36 that the root-specific reduction of CK perception would enhance shoot growth by increasing the amount

37 of root-derived tZ-type CKs and their perception by the shoot. This study will present a novel approach to

38 improve plant growth and productivity.

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Introduction

Cytokinins (CKs) regulate various developmental processes and environmental responses, including cell division, shoot and root growth, cambial proliferation, and stress responses (Werner et al. 2001, Riefler et al. 2006, Sakakibara 2006, Matsumoto-Kitano et al. 2008). N^6-(Δ^2-isopentenyl) adenine (iP) and *trans*-zeatin (tZ) are biologically active forms of CKs in *Arabidopsis thaliana*. The initial rate-limiting step of their biosynthesis is the N^6-prenylation of adenosine 5′-phosphates catalyzed by adenosine phosphate-isopentenyl transferases (IPTs), which produce iP-riboside 5′-phosphates (iPRPs) (Miyawaki et al. 2006, Osugi and Sakakibara 2015). Then, the iPRPs are converted into tZ-riboside 5′-phosphates (tZRPs) by two cytochrome P450 enzymes, CYP735A1, and CYP735A2 (Takei et al. 2004, Kiba et al. 2013). Finally, the iP-riboside 5′-monophosphate (iPRMP) and tZ-riboside (tZRMP) are dephosphoribosylated by the CK-activating enzyme LONELY GUY (LOG), which forms iP and tZ (Kurakawa et al. 2007, Kuroha et al. 2009, Tokunaga et al. 2012). They are perceived by three CK receptors, Arabidopsis HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4/CRE1 (Inoue et al. 2001, Higuchi et al. 2004, Nishimura et al. 2004), activating the CK signaling cascade.

Compelling evidence indicates that CKs function as a long-distance signal transported through the vasculature (Sakakibara 2021). ATP-binding cassette transporter subfamily G14 (ABCG14) is expressed predominantly in the stele of roots and facilitates root-to-shoot transport of CKs (Ko et al. 2014, Zhang et al. 2014). Reciprocal grafting between wild-type and *abcg14* has demonstrated that CKs transported from roots contribute to shoot growth. The CYP735A1 and CYP735A2 are also predominantly expressed in roots (Takei et al. 2004), and thus, tZ-type CKs are biosynthesized mainly in the root. Shoot-specific impairment in the growth of *cyp735a1 chp735a2* mutants indicates that root-derived tZ-type CKs are crucial for shoot growth (Kiba et al. 2013). Grafting between various mutants on CK biosynthesis and

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67   transport mutants has demonstrated that root-derived tZ, a minor component of xylem CKs, controls leaf

68   size but not the activity of the shoot apical meristem. By contrast, root-derived *trans*-zeatin riboside (tZR)

69   contributes to both traits (Osugi et al. 2017). A recent study has reported that root-derived tZ-type CKs

70   protect Arabidopsis shoots against photoperiod stress, abiotic stress caused by prolongation of the light

71   period (Frank et al. 2020).

72   As mentioned above, root-derived CKs enhance growth, development, and stress tolerance in

73   Arabidopsis shoots. This provoked us to produce plants whose shoots receive more CKs transported from

74   roots, thereby showing better shoot growth. It is worthy of attention that deficiency of CK receptors

75   significantly increases concentrations of tZ-type CKs in *A. thaliana*. By contrast, it impairs shoot growth

76   because of a lack of CK perception in the shoot (Riefler et al. 2006). Thus, we supposed that root-specific

77   reduction of CK perception could lead to CK accumulation in the root and facilitate their root-to-shoot

78   transport, enhancing shoot growth. To demonstrate this, we grafted Arabidopsis Col-0 (WT) scion onto

79   rootstock originated from WT or a double-knockout line of *AHK2* and *AHK3*. The grafted plants were

80   cultivated to analyze their growth, CK concentrations, and expression of CK-related genes. Here we show

81   that root-specific reduction of CK perception significantly enhances shoot growth and increases the levels

82   of tZ-type CKs in the shoot. The possible underlying mechanisms are discussed.

83

84   **Results**

85   *Confirmation of root-specific deficiency of AHK2 and AHK3*

86   To achieve the root-specific reduction of CK perception, we aseptically grafted WT scion onto rootstock

87   originated from WT or a double-knockout line of *AHK2* and *AHK3* using 6-day-old seedlings (Fig. 1A). We

88   used *ahk2-5 ahk3-7* (*ahk23*) as the knockout line because this line contains the most tZ-type CKs among all

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89   double-knockout lines on *AHK2/3/4* and has vigorous roots for successful grafting (Riefler et al. 2006). At

90   approximately 1 week after surgery, the grafted plants (scion/rootstock: WT/WT and WT/*ahk23*) having

91   vigorous growth without any adventitious roots were selected for further experiments. The genomic

92   polymerase chain reaction (PCR) and reverse transcription (RT)-PCR using the primers specific to *AHK2*,

93   *AHK3*, T-DNA left border, and *ACTIN2* confirmed root-specific deficiency of *AHK2* and *AHK3* in the

94   WT/*ahk23* plants (Fig. 1B, C).

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96   *Root-specific deficiency of AHK2 and AHK3 enhances shoot growth*

97   The grafted plants were transferred to the vermiculite pots to analyze the effects of root-specific

98   deficiency of CK perception on shoot growth. Then, the grafted plants were cultivated for 10 days under

99   10 mM nitrate (high N) or 0.5 mM nitrate (low N) because nitrogen availability is a strong determinant of

100   CK contents in *A. thaliana* (Sakakibara et al. 2006). We observed their apparent shoot growth and

101   determined the fresh weight, dry weight, rosette diameter, leaf area, and leaf number in their shoots (Fig.

102   2A–F). The WT/*ahk23* plants had larger shoot growth than did the WT/WT plants under both nitrate

103   conditions (Fig. 2A). The fresh shoot weight and dry weight were greater in the WT/*ahk23* plants than in

104   the WT/WT plants by 21% and 29% under high N and by 18% and 35% under low N on average,

105   respectively (Fig. 2B, C). There were significant increases in the rosette diameter and leaf area in the

106   WT/*ahk23* plants relative to the WT/WT plants (Fig. 2D, E). Alternatively, the leaf number was

107   comparable between the two types of grafted plants (Fig. 2F). *In vitro* culture of the grafted plants on the

108   solid media also confirmed that fresh shoot weight was significantly larger in the WT/*ahk23* plants than

109   in the WT/WT plants by 18% under high N and 20% under low N averagely (Supplementary Fig. S1A).

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111   *Root-specific deficiency of AHK2 and AHK3 does not affect the root growth*

112   It has been widely reported that the length and number of lateral roots are increased by a deficiency of

113   CK biosynthesis, perception, and signaling (Miyawaki et al. 2006, Riefler et al. 2006). However, in this

114   study, no significant difference was observed in the total root length and lateral root number between the

115   WT/WT and WT/*ahk23* plants vertically grown on solid media containing 10 or 0.5 mM nitrate (Fig. 3A,

116   B). Also, the fresh root weight was comparable between the horizontally grown grafted plants

117   (Supplementary Fig. S1B).

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119   *Root-specific deficiency of AHK2 and AHK3 increases root tZ-type CK levels, induces CK biosynthesis*

120   *genes, and represses CK degradation genes*

121   Next, to elucidate the effects of root-specific deficiency of CK perception on the root CK levels, we

122   determined tZ-type and iP-type CKs in the root of grafted plants. Not only concentrations of tZ and tZR

123   but also those of the other tZ-type CKs and iP-type CKs were consistently higher in the WT/*ahk23* roots

124   than in the WT/WT roots under both nitrate regimes (Fig. 4A–E and Supplementary Fig. S2A–D). The tZ

125   and tZR concentrations in the WT/*ahk23* roots were averagely 2.8 and 1.9 times under high N and 2.7 and

126   2.3 times under low N as much as those in the WT/WT roots, respectively (Fig. 4A, B). The

127   concentrations of respective CK species were generally larger under the 10 mM nitrate conditions than

128   under the 0.5 mM nitrate conditions (Fig. 4A–E and Supplementary Fig. S2A–D).

129   To analyze how root-specific deficiency of CK perception increases the root CK levels, we

130   performed quantitative RT-PCR analysis on the genes of the *CYTOKININ*

131   *OXIDASE/DEHYDROGENASEs* (*CKXs*) involved in CK degradation and the *IPTs* and *CYP735A1/A2*

132   contributing to *de novo* biosynthesis of iP-type CKs and their conversion to tZ-type CKs. While the

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133   expression of *CKXs* except for *CKX3* was downregulated by root-specific deficiency of *AHK2* and *AHK3*,

134   that of *IPT1*, *IPT3*, *IPT5*, and *IPT7* was upregulated (Fig. 5). Notably, the transcript levels of *CKX2* and

135   *CKX4* were lower in the WT/*ahk23* roots than in the WT/WT roots by 93% and 91% under high N and

136   95% and 84% under low N on average, respectively (Fig. 5). The opposite changes in *IPTs* and *CKXs*

137   expression would collectively enhance CK accumulation in roots deficient in CK perception. The root

138   expression of *CYP735A2* was comparable between the grafted plants under high N conditions, whereas

139   that was larger in the WT/*ahk23* plants than in the WT/WT plants under low N conditions (Fig. 5).

140   Irrespective of N conditions, the expression of *CYP735A1*, a minor isogene, was higher in the WT/*ahk23*

141   roots than in the WT/WT roots (Fig. 5). These suggest that root-specific deficiency of CK perception

142   could upregulate the root genes contributing to tZ-type CK biosynthesis, especially under low N

143   conditions. The expression of *ABCG14* involved in the root-to-shoot transport of tZ-type CKs was little

144   changed depending on the grafting (Fig. 5).

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146   *Root-specific deficiency of AHK2 and AHK3 increases tZ-type CK levels and expression of CK-inducible*

147   *genes in the shoot*

148   To analyze the effects of root-specific deficiency of CK perception on the shoot CK levels, we

149   determined tZ-type and iP-type CKs in the shoots of grafted plants. Shoot concentrations of tZ-type CKs

150   were consistently higher in the WT/*ahk23* plants (Fig. 6A–C and Supplementary Fig. S3A, B). The tZ

151   and tZR concentrations in the WT/*ahk23* shoots were averagely 1.4 and 1.7 times under high N and 1.4

152   and 1.5 times under low N as much as those in the WT/WT shoots, respectively (Fig. 6A, B). By contrast,

153   shoot concentrations of iP-type CKs were not always higher in the WT/*ahk23* plants than in the WT/WT

154   plants (Fig. 6D, E and Supplementary Fig. S3C, D). These imply that the root-to-shoot transport of

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155   xylem-mobile tZ and tZR could be larger in the WT/*ahk23* plants than in the WT/WT plants. The shoot

156   concentrations of tZ, tZR, tZRPs, iPR, and iPRPs were generally larger under high N than under low N in

157   the same way as the roots (Fig. 6A–E), whereas those of cytokinin *N*- and *O*-glucosides were similar

158   under both N conditions (Supplementary Fig. S3A, B, D).

159   To verify whether the increased tZ-type CKs stimulate CK signaling in the WT/*ahk23* shoots,

160   we checked the gene expression of CK-inducible type-A *ARABIDOPSIS RESPONSE REGULATORS*

161   (*ARRs*) (Osugi et al. 2017). The expression of the *ARRs* was upregulated in the WT/*ahk23* shoots

162   compared with the WT/WT shoots, and upregulation was pronounced under low N conditions compared

163   with high N conditions (Fig. 7). This suggests that root-specific deficiency of CK perception would

164   enhance CK signaling in the shoot.

165

166   **Discussion**

167   CK signaling enhances the growth and development of shoots, whereas negatively regulates those of

168   roots (Miyawaki et al. 2006, Riefler et al. 2006, Osugi et al. 2017). Consequently, the deficiency of genes

169   for CK biosynthesis, perception, and signaling leads to relatively smaller shoots and larger roots.

170   Interestingly, root-specific reduction of CK contents by constitutively expressing *CKX* under the control

171   of root-specific promoter enhances root growth and mineral absorption efficiency apparently without

172   impairing vegetative shoot growth in *A. thaliana* and tobacco (Werner et al. 2010). Similar approaches

173   have succeeded in improving root growth and crop yields in barley and chickpea (Ramireddy et al. 2018,

174   Khandal et al. 2020). On the other hand, root-specific expression of *CKX* significantly decreases the shoot

175   concentrations of tZ-type and iP-type CKs, albeit with no negative effects on shoot growth (Werner et al.

176   2010). Since the tZ-type CKs transported from roots promote shoot growth (Kiba et al. 2013, Osugi et

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177   2017), this study aimed to increase the amounts of root-derived CKs by root-specific reduction of CK

178   perception. Expectedly, CKs accumulated in the roots lacking AHK2 and AHK3 (Fig. 4 and

179   Supplementary Fig. S2), probably because of the induction of *IPTs* and repression of *CKXs* (Fig. 5).

180   Shoot growth, shoot concentrations of tZ-type CKs, and shoot expression of CK-inducible marker genes

181   were consistently increased in the WT/*ahk23* plants compared with that in the WT/WT (Fig. 2, 6A–C, 7,

182   and Supplementary Fig. S1A, S3A, B). Previous studies have demonstrated that tZ-type CKs are

183   predominantly produced from iP-type CKs in the root (Kiba et al. 2013) and tZ and tZR are xylem-mobile

184   from root-to-shoot (Osugi et al. 2017). Also, we observed that the shoot expression of *CYP735A2* was

185   approximately 1% as much as its root expression and was not significantly induced by the root-specific

186   deficiency of *AHK2* and *AHK3* (Supplementary Fig. S5). The *CYP735A1* expression was not detected in

187   the shoot (data not shown). These indicate that the increased tZ-type CKs in the WT/*ahk23* shoots could

188   originate from their roots. Kiba et al. (2013) have reported that *cyp735a1 cyp735a2* mutants lacking the

189   tZ-type CKs grew rosette leaves with reduced leaf blade size and petiole length compared with WT plants,

190   whereas their leaf number was not altered. In this study, root-specific reduction of CK perception

191   increased leaf area and rosette diameter but not leaf number (Fig. 2), which indicates the increased action

192   of tZ-type CKs. Moreover, we observed that the root-specific deficiency of *AHK2* and *AHK3* enhanced

193   shoot growth in the WT scion more effectively than in the *ahk23* scion except for the rosette diameter

194   under high N (Fig. 2A and Supplementary Fig. S4), suggesting that a significant part of shoot growth

195   enhancement would occur through increased shoot perception of CKs. Altogether, we conclude that the

196   root-specific reduction of CK perception would enhance shoot growth by increasing the amounts of

197   root-derived tZ-type CKs and their perception by the shoot.

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198   The root-specific deficiency of *AHK2* and *AHK3* induced the shoot expression of

199   CKs-inducible *ARRs* more intensively under low N than under high N except for *ARR5* and *ARR16* (Fig.

200   7). The basal levels of tZ, tZR, and tZRPs in shoots were generally lower under low N than under high N

201   (Fig. 6A–C). Hence, the increase in root-derived CKs could drive the CK signaling more effectively in the

202   shoots lacking CKs than those filled with CKs. On the other hand, the root-specific *AHK2* and *AHK3*

203   deficiency enhanced shoot growth almost equally between the two N regimes (Fig. 2 and Supplementary

204   Fig. S1). Thus, in the shoot, the increase in CK signaling is not always correlated with growth

205   enhancement, implying that the unknown signaling molecule(s) other than CKs might be also involved in

206   the growth enhancement.

207   Kiba et al. (2011) reported that under low N conditions, exogenous application of tZ

208   significantly represses the expression of high-affinity nitrate transporters *NRT2.1* and *NRT2.4*, depending

209   on the functional AHK3 and AHK4/CRE1. This implies that root-specific reduction of CK perception

210   could elevate *NRT2.1* and *NRT2.4* expression, thereby facilitating nitrate acquisition, especially under low

211   N conditions. However, the transcript levels of *NRT2.1* and *NRT2.4* were not significantly different

212   between the roots of WT/WT and WT/*ahk23* regardless of the N regime (Supplementary Fig. S6A, B).

213   Also, the nitrate concentrations in the shoot and the root did not significantly differ between the grafted

214   plants (Supplementary Fig. S6C, D). It awaits further challenges by the root-specific deficiency of *AHK3*

215   and *AHK4* or *AHK2* and *AHK3* and *AHK4* to improve N uptake ability.

216   Although root-specific reduction of CK contents strongly enhances root growth and

217   development in previous studies (Werner et al. 2010, Ramireddy et al. 2018, Khandal et al. 2020), our

218   root-specific reduction of CK perception did not significantly alter them (Fig. 3 and Supplementary Fig.

219   S1B). In this study, the plants before and after grafting were cultivated under relatively low-light

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220   conditions (photosynthetic photon flux density [PPFD]: approximately 40–50 μmol m^{-2} s^{-1}) to alleviate

221   photooxidative stress under low N conditions (Cetner et al. 2017). Therefore, the shortage of carbon

222   sources and additional carbon consumption by shoot growth enhancement might limit the biomass

223   allocation to the WT/*ahk23* root. In the future study, we will test whether higher light intensities or

224   carbon dioxide concentrations improve root growth.

225   In the present study, we have succeeded in increasing shoot growth by root-specific reduction

226   of CK perception. Nowadays, the genome editing technologies are applicable for disrupting a gene of

227   interest in various plant species and therefore, the rootstocks lacking CK receptors would be available in

228   useful crops. This study presents a novel approach to improve crop growth and productivity.

229

230   **Materials and Methods**

231   *Plant materials and growth conditions*

232   Seeds of Col-0 and *ahk2-5 ahk3-7* (Riefler et al. 2006) were surface-sterilized and sown in the solid

233   medium containing half-strength Murashige and Skoog (1/2MS) salts, supplemented with 0.05% (w/v)

234   MES-KOH (pH 5.7), 1% (w/v) sucrose, and 0.5% (w/v) gellan gum (Fujifilm Wako, Osaka, Japan). The

235   seeds were placed in the dark at 4°C for 3–5 days to break dormancy. Seedlings were grown vertically

236   under a PPFD of 25 μmol m^{-2} s^{-1} (16 h light/8 h dark cycle) at 22°C. The micrografting was aseptically

237   performed using the 6-day-old seedlings. The seedlings were cut perpendicularly in the hypocotyl with an

238   injection needle tip (Code NN-2613S; TERUMO, Tokyo, Japan). The obtained scion was connected with

239   the partner rootstock in a silicon microtube (Code 1-8194-04; AS ONE, Osaka, Japan). The grafted plants

240   were incubated for 5 days under a PPDF of approximately 40–50 μmol m^{-2} s^{-1} (constant light) at 27℃

241   and further cultivated for a few days under a PPFD of approximately 25 μmol m^{-2} s^{-1} (16 h light/8 h dark

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242   cycle) at 22°C. The successfully grafted plants were selected, transferred to vermiculite pots or solid

243   media, and grown for the following experiments. For vermiculite cultivation, the plants were grown under

244   a PPFD of approximately 40–50 μmol m^{-2} s^{-1} (16 h light/8 h dark cycle) at 22°C. The 30 mL of

245   nitrogen-modified MGRL-based salts (Fujiwara et al. 1992); including 10 mM potassium nitrate (high N)

246   or 0.5 mM potassium nitrate and 9.5 mM potassium chloride (low N), was supplied twice before harvest.

247   For the *in vitro* culture on the solid media, the plants were grown under a PPFD of approximately 40–

248   50 μmol m^{-2} s^{-1} (constant light) at 22°C in the nitrogen-modified 1/2MS salts including 10 mM

249   potassium nitrate (high N) or 0.5 mM potassium nitrate and 9.5 mM potassium chloride (low N)

250   supplemented with 0.1% (w/v) MES-KOH (pH 5.7), 1% (w/v) sucrose, and 0.25% (horizontally) or 0.5%

251   (vertically) (w/v) gellan gum (Wako, Osaka, Japan). To allow nondestructive sampling of the roots in

252   horizontally grown plants, the surfaces of solid media were covered with cellophane sheets (Hachiya et al.

253   2021). The other details are described in the figure legends.

254

255   *Extraction of total RNA*

256   Shoots and roots were harvested, immediately frozen with liquid N$_2$, and stored at −80°C until use. Shoots

257   and roots from five plants were pooled as one biological replicate. Frozen samples were ground with a

258   Multi-Beads Shocker (Yasui Kikai Corp., Osaka Prefecture, Osaka, Japan) using zirconia beads (diameter,

259   5 mm). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen Japan, Tokyo, Japan)

260   according to the manufacturer's instructions.

261

262   *Quantitative RT-PCR*

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263   Reverse transcription was performed using a ReverTra Ace qPCR RT Master Mix with gDNA Remover

264   (Toyobo Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The synthesized cDNA

265   was diluted fivefold with distilled water and used in quantitative PCR (qPCR). Transcript levels were

266   measured using a TaKaRa Thermal Cycler Dice TP800 (TaKaRa Bio Inc., Shiga, Japan). The obtained

267   cDNA (2 µL) was amplified in the presence of 10 µL KOD SYBR qPCR Mix (TOYOBO Co. Ltd.),

268   0.4 µL specific primers (0.2 µM final concentration), and 7.2 µL sterile distilled water. Relative transcript

269   levels were calculated using the comparative cycle threshold (Ct) method using *ACTIN3* as the internal

270   standard (Hachiya et al. 2020). The primer sequences used in the experiments are shown in

271   Supplementary Table S1.

272

273   *Determination of CK species*

274   Shoots and roots were snap-frozen with liquid N_2 and stored at −80°C until use. Shoots and roots from

275   five plants were pooled as one biological replicate. Extraction and determination of CKs were performed

276   as described previously (Kojima et al. 2009); using ultra-performance liquid chromatography (UPLC)–

277   tandem quadrupole mass spectrometry (AQUITY UPLC System/XEVO-TQS; Waters) with an

278   octadecylsilyl column (ACQUITY UPLC HSS T3, 1.8 µm 2.1 mm × 100 mm).

279

280   *Determination of nitrate*

281   Nitrate was determined according to the method reported by Hachiya and Okamoto (2017). Shoots and

282   roots were snap-frozen with liquid N_2 and stored at −80℃ until use. Shoots and roots from independent

283   plates were pooled separately as single biological replicates for samples. The shoots and roots were boiled

284   with 10 volumes of H_2O at 100°C for 20 min. The obtained 10 µL supernatant or the 10 µL standard

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285 solution of potassium nitrate was mixed with a 40 μL reaction reagent containing 50 mg salicylic acid per

286 1 mL sulfuric acid, followed by incubation at room temperature for 20 min. For the mock preparation,

287 40 μL sulfuric acid alone instead of the reaction reagent was added to the 10 μL supernatant. After adding

288 1 mL of 8% (w/v) NaOH solution to the mixture, the absorbance at 410 nm was scanned using a plate

289 reader (CORONA SH-9000Lab, Hitachi High-Tech Corp., Tokyo, Japan). The nitrate concentration was

290 calculated from the standard curve.

291

292 *Statistical*

293 Statistical analyses were done using the R software v.2.15.3.

294

306

307   **Disclosures**

308   Conflicts of interest: No conflicts of interest declared

309

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387

388   **Legends to figures**

389   **Fig. 1** Root-specific deficiency of *AHK2* and *AHK3* by micrografting technique. (A) A schematic

390   representation of two types of grafted plants. WT; wild-type Col-0, *ahk23*; *ahk2-5 ahk3-7* (Riefler et al.

391   2006). The dotted line represents the grafted seam in the hypocotyl. (B) A representative result of

392   genomic PCR and agarose electrophoresis to confirm root-specific deficiency of *AHK2* and *AHK3*.

393   *AHK2-F1*, *AHK2-R1*, *AHK3-F1*, *AHK3-R1*, *LB1*, and *LB2* are the specific primers used for PCR

394   (Supplementary Table 1). (C) A representative result of RT-PCR and agarose electrophoresis to confirm

395   root-specific deficiency of *AHK2* and *AHK3*. AHK2-F2, AHK2-R2, AHK3-F2, AHK3-R2, and *ACTIN2*

396   are the specific primers used for PCR (Supplementary Table 1).

397

398   **Fig. 2** Effects of root-specific deficiency of *AHK2* and *AHK3* on shoot growth. The grafted plants were

399   transferred to the vermiculite pots and grown for 10 days with a supply of MGRL-based salts containing

400   10 mM (high N) or 0.5 mM (low N) nitrate. The shoot appearance (A), shoot fresh weight (B), shoot dry

401   weight (C), rosette diameter (D), leaf area (E), and leaf number (F) of the grafted plants were analyzed.

402   Data are presented as the mean ± SD (n = 11–12). WT/WT, WT/*ahk23*, *ahk23*/WT, and *ahk23*/*ahk23*

403   denote the plants corresponding to the WT scion, WT scion, *ahk23* scion, and *ahk23* scion grafted on the

404   WT rootstock, *ahk23* rootstock, WT rootstock, and *ahk23* rootstock, respectively. *$P < 0.05$; **$P < 0.01$;

405   ***$P < 0.001$ (Welch's *t*-test). NS denotes not significant. FW, fresh weight; DW, dry weight. The scale

406   bar denotes 1 cm.

407

408   **Fig. 3** Effects of root-specific deficiency of *AHK2* and *AHK3* on root growth. The grafted plants were

409   transferred to nitrogen-modified 1/2MS media containing 10 mM (high N) or 0.5 mM (low N) nitrate and

410   grown vertically for 3 days. The total root length (A) and lateral root number (B) of the grafted plants

411   were analyzed. Quantitative data are presented as the mean ± SD (n = 25–26). NS denotes not significant

412   (Welch's *t*-test).

413

414   **Fig. 4** Effects of root-specific deficiency of *AHK2* and *AHK3* on cytokinin concentrations in the root. The

415   grafted plants were transferred to nitrogen-modified 1/2MS media containing 10 mM (high N) or 0.5 mM

416   (low N) nitrate and grown in a horizontal position for 7 days. The samples harvested from four

417   independent grafting experiments were subjected to cytokinin analysis. '1', '2', '3', and '4' below the

418   graph mean the 1st, 2nd, 3rd, and 4th grafting experiment, respectively. Roots from five plants were

20

419   pooled as one biological replicate. The cytokinin analysis for the 1st and 2nd samples and that for the 3rd

420   and 4th samples were separately performed. The root concentrations of tZ (A), tZR (B), tZRPs (C), iPR

421   (D), and iPRPs (E) in the grafted plants are shown. tZ, *trans*-zeatin; tZR, tZ-riboside; tZRPs, tZR

422   5′-phosphates; iPR, N^6-(Δ^2-isopentenyl)adenine riboside; iPRPs, iPR 5′-phosphates.

423

424   **Fig. 5** Effects of root-specific deficiency of *AHK2* and *AHK3* on the expression of genes contributing to

425   cytokinin degradation, biosynthesis, and transport in the root. The grafted plants were transferred to

426   nitrogen-modified 1/2MS media containing 10 mM (high N) or 0.5 mM (low N) nitrate and grown in a

427   horizontal position for 7 days. The samples harvested from three independent grafting experiments were

428   subjected to total RNA purification and subsequent RT-qPCR. '1st', '2nd', and '3rd' mean the 1st, 2nd,

429   and 3rd grafting experiment, respectively. Roots from five plants were pooled as one biological replicate.

430   The relative transcript levels in the WT/*ahk23* roots are shown regarding those in the WT/WT roots as

431   one.

432

433   **Fig. 6** Effects of root-specific deficiency of *AHK2* and *AHK3* on cytokinin concentrations in the shoot.

434   The grafted plants were transferred to nitrogen-modified 1/2MS media containing 10 mM (high N) or 0.5

435   mM (low N) nitrate and grown in a horizontal position for 7 days. The samples harvested from four

436   independent grafting experiments were subjected to cytokinin analysis. '1', '2', '3', and '4' below the

437   graph mean the 1st, 2nd, 3rd, and 4th grafting experiment, respectively. Shoots from five plants were

438   pooled as one biological replicate. The cytokinin analysis for the 1st and 2nd samples and that for the 3rd

439   and 4th samples were separately performed. The shoot concentrations of tZ (A), tZR (B), tZRPs (C), iPR

21

440   (D), and iPRPs (E) in the grafted plants are shown. tZ, *trans*-zeatin; tZR, tZ-riboside; tZRPs, tZR

441   5′-phosphates; iPR, N^6-(Δ^2-isopentenyl) adenine riboside; iPRPs, iPR 5′-phosphates.

442

443   **Fig. 7** Effects of root-specific deficiency of *AHK2* and *AHK3* on the expression of cytokinin-inducible

444   type-A *ARR* genes in the shoot. The grafted plants were transferred to nitrogen-modified 1/2MS media

445   containing 10 mM (high N) or 0.5 mM (low N) nitrate and grown in a horizontal position for 7 days. The

446   samples harvested from three independent grafting experiments were subjected to total RNA purification

447   and subsequent RT-qPCR. 1st', '2nd', and '3rd' mean the 1st, 2nd, and 3rd grafting experiment,

448   respectively. Shoots from five plants were pooled as one biological replicate. The relative transcript levels

449   in the WT/*ahk23* shoots are shown regarding those in the WT/WT shoots as one.
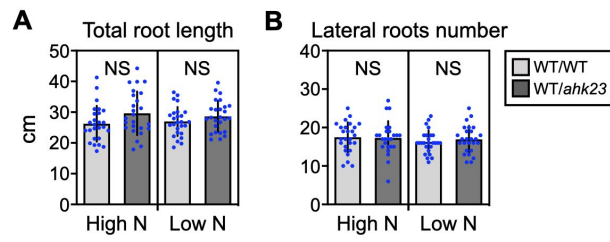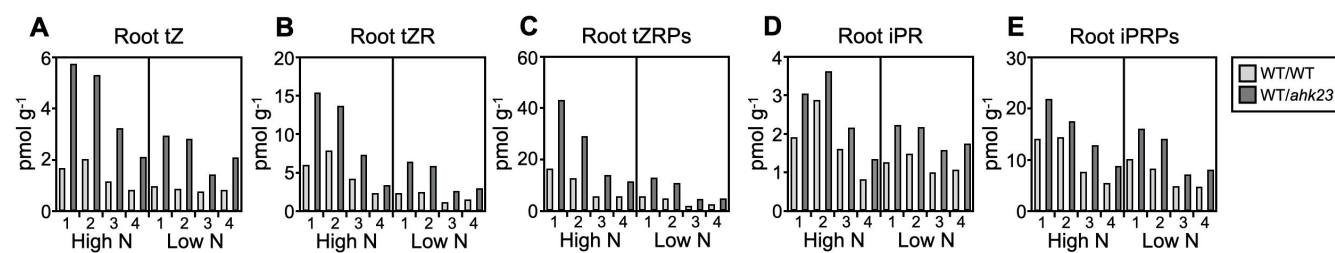
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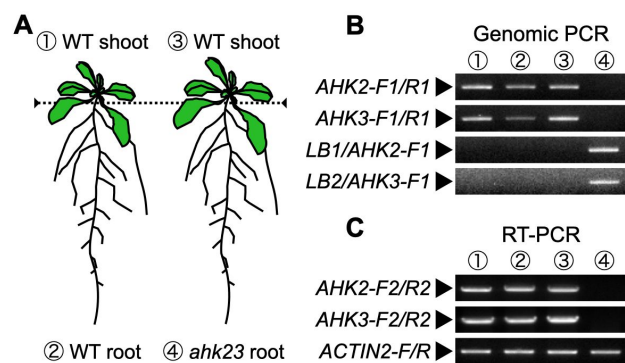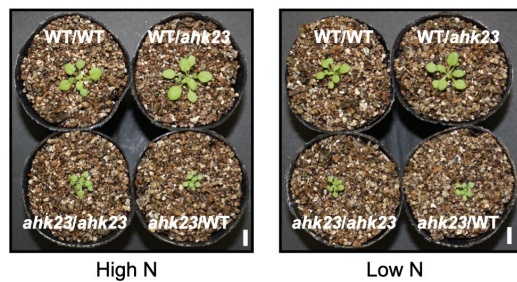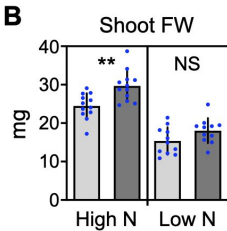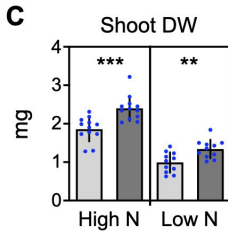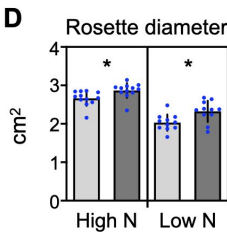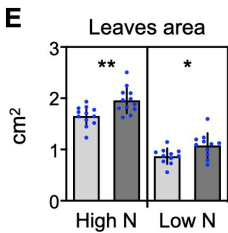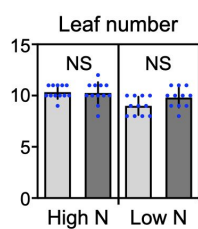
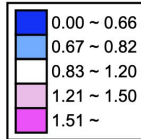**Figure 1**

Figure 2

Figure 3

Figure 4
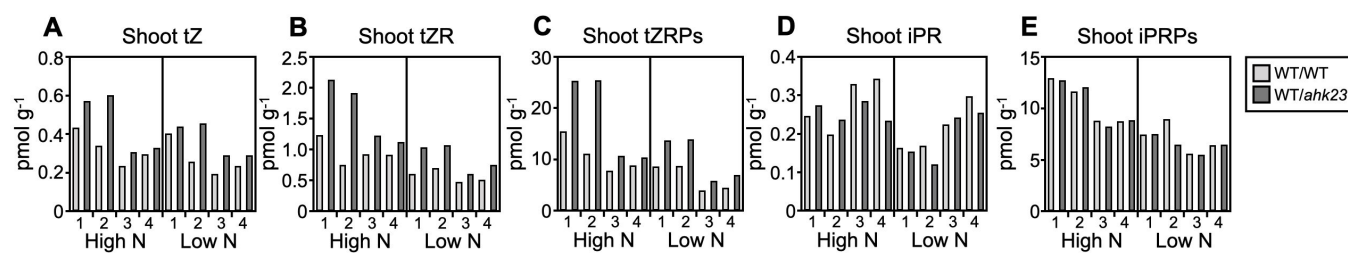
Figure 5

Fold change of gene expression
in WT/*ahk23* to WT/WT (Roots)

Gene Name	High N			Low N		
	1st	2nd	3rd	1st	2nd	3rd
CKX1	0.77	0.79	0.73	0.82	0.70	1.05
CKX2	0.07	0.09	0.06	0.07	0.05	0.04
CKX3	1.07	1.26	1.06	1.07	1.23	1.21
CKX4	0.09	0.09	0.08	0.15	0.20	0.14
CKX5	0.56	0.42	0.48	0.65	0.95	0.66
CKX6	0.80	0.77	0.72	0.86	0.81	0.90
CKX7	0.61	0.56	0.56	0.81	0.73	0.97
IPT1	1.04	1.05	1.48	1.19	1.36	1.73
IPT3	1.31	1.42	1.60	1.53	1.36	1.63
IPT5	1.57	1.91	1.53	1.55	0.96	1.46
IPT7	2.25	1.43	2.56	1.66	1.12	1.93
CYP735A1	1.41	1.27	1.14	1.34	1.18	1.66
CYP735A2	0.91	0.92	0.86	1.33	1.34	1.57
ABCG14	0.91	0.85	0.90	1.07	1.04	1.07

Legend:
- 0.00 ~ 0.66
- 0.67 ~ 0.82
- 0.83 ~ 1.20
- 1.21 ~ 1.50
- 1.51 ~

Figure 6

Figure 7

Fold change of gene expression
in WT/*ahk23* to WT/WT (Shoots)

Gene Name	High N			Low N		
	1st	2nd	3rd	1st	2nd	3rd
ARR4	1.07	1.21	1.16	1.54	1.35	1.34
ARR5	1.22	1.70	1.18	1.05	1.15	1.02
ARR6	1.14	1.36	1.15	1.76	1.51	1.14
ARR7	0.88	0.89	0.90	2.10	1.97	1.27
ARR8	1.05	1.03	1.16	1.04	0.95	0.82
ARR9	1.10	1.26	1.04	1.31	1.23	0.99
ARR15	0.89	1.16	0.74	3.92	7.19	2.75
ARR16	1.50	1.27	2.12	1.46	0.73	1.43

	0.00 ~ 0.66
	0.67 ~ 0.82
	0.83 ~ 1.20
	1.21 ~ 1.50
	1.51 ~