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Suppression of the *Arabidopsis* cinnamoyl-CoA reductase 1-6 intronic T-DNA mutation by epigenetic modification

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1 **Short title:** *trans* T-DNA suppression of the *ccr1-6* allele

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9

10 **Suppression of the Arabidopsis *cinnamoyl-CoA***
11 ***reductase 1-6* intronic T-DNA mutation by epigenetic**
12 **modification**

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24 M.W., R.V. and W.B. designed the study and analyzed the data; G.N. made the *ugt72e1,-*
25 *e2,-e3* mutant. L.d.V. performed the *ccr1-6 x ugt72e1,-e2,-e3* cross and isolated the
26 quadruple mutant; M.W. and H.B. extracted the genomes, M.W. and S.R. performed the
27 ONT sequencing of the *ccr1-6* and *ugt72e1,-e2,-e3 epiccr1-6* mutants. T.D.P. and M.S.
28 performed the ONT sequencing of the *ugt72e1,-e2,-e3* mutant. S.R. analyzed the ONT
29 sequencing data; H.B. analyzed the DNA methylation and made the respective figures; M.W.
30 isolated *epiccr1-6* and performed all other experiments; M.W., R.V. and W.B. wrote the
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37 **One sentence summary:** The dwarfed phenotype of the *Arabidopsis ccr1-6* intronic T-DNA
38 lignin mutant is restored by the introduction of additional T-DNAs sharing sequence
39 homology.

40 **Abstract**

41 *Arabidopsis thaliana* T-DNA insertion collections are popular resources for fundamental plant
42 research. Cinnamoyl-CoA reductase 1 (CCR1) catalyzes an essential step in the
43 biosynthesis of the cell wall polymer lignin. Accordingly, the intronic transfer (T)-DNA
44 insertion mutant *ccr1-6* has reduced lignin levels and shows a stunted growth phenotype.
45 Here, we report restoration of the *ccr1-6* mutant phenotype and *CCR1* expression levels after
46 a genetic cross with a *UDP-glucosyltransferase 72e1 (ugt72e1)*,-e2,-e3 T-DNA mutant. We
47 discovered that the phenotypic recovery was not dependent on the UGT72E family loss of
48 function but due to an epigenetic phenomenon referred to as *trans* T-DNA suppression. Via
49 *trans* T-DNA suppression, the gene function of an intronic T-DNA mutant is restored after
50 introduction of an additional T-DNA sharing sequence homology, leading to
51 heterochromatinization and splicing out of the T-DNA-containing intron. Consequently, the
52 suppressed *ccr1-6* allele was named *epiccr1-6*. Long-read sequencing revealed that *epiccr1-*
53 *6*, and not *ccr1-6*, carries dense cytosine methylation over the full-length of the T-DNA. We
54 show that the SAIL T-DNA in the *UGT72E3* locus could trigger the *trans* T-DNA suppression
55 of the GABI-Kat T-DNA in the *CCR1* locus. Furthermore, we scanned the literature for other
56 potential cases of *trans* T-DNA suppression in *Arabidopsis*. These combined observations
57 indicate that intronic T-DNA mutants need to be used with caution, since methylation of
58 intronic T-DNA might derepress gene expression and can thereby confound results.

59 **Introduction**

60 Mutants are extensively used in fundamental plant research. In the model plant *Arabidopsis*
61 *thaliana* (*Arabidopsis*), more than 260,000 transfer (T)-DNA insertion lines are available in
62 the Columbia-0 accession (O'Malley et al., 2015). These lines were generated by random
63 *Agrobacterium*-mediated T-DNA integration in the genome: T-DNA insertion in an exon
64 causes interruption of the coding sequence, whereas T-DNA insertion in an intron can
65 interfere with proper mRNA splicing. GABI-Kat (Rosso et al., 2003; Kleinboelting et al.,

66 2012), SALK (Alonso et al., 2003) and SAIL (Sessions et al., 2002) are the T-DNA consortia
67 that make up the bulk of the mutant lines and differ each in the elements encoded on the T-
68 DNA. A different source of mutants (48,000 lines) is provided by the John Innes Centre (JIC)
69 Suppressor-mutator (SM) transposant library (Tissier et al., 1999). Initially, this library was
70 built by T-DNA integration, after which a defective Suppressor-mutator (dSpm) element
71 jumped out of the T-DNA sequence by means of a transposase also encoded by the T-DNA.
72 The final JIC SM transposon lines are free of T-DNA sequences.

73 As for many other biological processes, unraveling the biosynthesis of lignin involved
74 ample use of T-DNA insertion mutants (Sibout et al., 2003; Vanholme et al., 2012b; Van
75 Acker et al., 2013; Wang et al., 2022). Lignin is a complex phenolic polymer in the plant
76 secondary cell wall that confers both fiber strength, to allow plants to grow upright, and
77 vessel hydrophobicity, to enable water transport. However, lignin is also the major factor
78 determining recalcitrance to biomass processing into pulp and fermentable sugars in the
79 biorefinery and, thus, plants are engineered to produce less lignin (Chen and Dixon, 2007;
80 Ralph et al., 2019; Vanholme et al., 2019). Unfortunately, low-lignin plants often suffer from a
81 yield penalty, making them less useful for industrial applications (Bonawitz et al., 2014; De
82 Meester et al., 2018; Muro-Villanueva et al., 2019; Gallego-Giraldo et al., 2020; Panda et al.,
83 2020). Therefore, engineering low-lignin biomass crops without yield penalty is an active field
84 of research (Chanoca et al., 2019; De Meester et al., 2020; 2021; 2022).

85 Lignin is synthesized from phenylalanine through the general phenylpropanoid and
86 monolignol-specific pathways (**Figure 1**), and is mainly made from the *p*-coumaryl, coniferyl
87 and sinapyl alcohols (Vanholme et al., 2012a; Ralph et al., 2019; Vanholme et al., 2019).
88 Oxidative coupling of these monolignols in the cell wall gives rise to the *p*-hydroxyphenyl (H),
89 guaiacyl (G) and syringyl (S) units of the lignin polymer, respectively. In lignifying cells, also a
90 small fraction of ferulic acid (FA) is translocated to the cell wall where it is incorporated into
91 the lignin polymer as a FA unit (Ralph et al., 2008; Leplé et al., 2007; Van Acker et al., 2013).
92 Nonetheless, the bulk of FA is metabolized into FA derivatives such as FA 4-*O*-glucoside and
93 feruloyl glucose and stored in the vacuole (Bowles et al., 2006; Vanholme et al., 2012b; Dima
94 et al., 2015; Le Roy et al., 2016).

95 Cinnamoyl-CoA reductase 1 (CCR1) catalyzes the first step of the monolignol-specific
96 pathway (**Figure 1**). The Arabidopsis GABI-Kat intronic T-DNA insertion mutant *ccr1-6* (GK_
97 622C01) shows a 52% reduction in lignin content and a plant biomass yield penalty of 34%
98 (Van Acker et al., 2013). The stunted growth of *ccr1-6* mutants is most likely due to the lack
99 of lignin in the vessels, because vessel-specific reintroduction of *CCR1* expression in *ccr1-6*
100 mutants restores the plant biomass yield without reinstating the overall inflorescence stem
101 lignin content to wild-type levels (De Meester et al., 2018). *ccr1-6* mutants also produce large
102 amounts of FA (the hydrolysis product of CCR1's substrate feruloyl-CoA; **Figure 1**), resulting

103 in a relatively modest increase in FA units in lignin and a severe increase in FA sugar-
104 derivatives in the vacuole (Vanholme et al., 2012a; De Meester et al., 2018).

105 The uridine diphosphate (UDP)-glucosyltransferase 72E (UGT72E) gene family,
106 consisting of three members in Arabidopsis, is involved in the glucosylation of
107 phenylpropanoids (Lim et al., 2001; Lim et al., 2005; Lanot et al., 2006; Speeckaert et al.,
108 2020). More in particular, UGT72E2 has been shown to catalyze the glucosylation of FA to
109 its respective 4-O-glucoside *in vitro* (Lim et al., 2001; Lim et al., 2005) and three FA-derived
110 glucosides were identified as products of UGT72Es *in vivo* (Wu et al., 2022) (**Figure 1**). In
111 the present study, we attempted to overcome the biomass yield penalty of *ccr1-6* by
112 generating a quadruple mutant from a cross between *ccr1-6* and the *ugt72e1* (GK_340H02),-
113 *e2* (SM_3_20654),-*e3* (SAIL_1279_D02) T-DNA mutant. According to our working
114 hypothesis, blocking 4-O-glucosylation of FA by knocking out the *UGT72E* gene family would
115 prevent the storage of those FA derivatives in the vacuole and, in turn, increase the amount
116 of FA that can be incorporated as FA units in the lignin polymer. As such, compensating for
117 the deficit in traditional lignin units could potentially restore the growth of the *ccr1-6* mutants.
118 In addition to strengthening the cell wall, the increased amount of FA units in the lignin
119 polymer would also make lignin easier to degrade, because its incorporation introduces
120 acetal functionalities in the polymer that are readily cleavable in mild acid conditions (Ralph
121 et al., 2008; Van Acker et al., 2013).

122 Here, we report the complete restoration of the *ccr1-6* mutant phenotype after a genetic
123 cross with the *ugt72e1,-e2,-e3* T-DNA mutant. However, the suppression was not caused by
124 knocking out the *UGT72E* genes, but rather the result of *trans* T-DNA suppression.

125 **Results**

126 **The *ccr1-6* stunted growth is completely restored after a genetic cross with the** 127 ***ugt72e1,-e2,-e3* mutant**

128 Homozygous *ugt72e1,-e2,-e3* and homozygous *ccr1-6* mutants were crossed to study
129 whether blocking 4-O-glucosylation of FA by knocking out the *UGT72E* gene family could
130 result in the restoration of the growth phenotype of *ccr1-6* mutants. As envisioned, the *ccr1-6*
131 phenotype was fully recovered in the homozygous *ugt72e1,-e2,-e3 ccr1-6* mutant
132 (**Figure 2A, Supplemental Table S1**). However, we noted a deviation from the Mendelian
133 segregation in the F2 progeny of the original cross, as not a single plant out of the 480 F2
134 plants had the *ccr1-6* phenotype. Nevertheless, given that *ugt7e1,-e2* and *-e3* segregate
135 independently, 27/256 of the F2 plants were expected to be homozygous for the *ccr1-6*
136 mutation and to have at least one wild-type allele of each *UGT72E1, -E2* and *-E3*. To further

137 investigate this observation, homozygous *ccr1-6* mutants lacking the *ugt72e1,-e2* and *-e3*
138 mutations were reisolated from the F3 progeny. Interestingly, the dwarfed phenotype of the
139 parental *ccr1-6* was absent in the reisolated *ccr1-6* mutant homozygous for the *CCR1* T-DNA
140 insertion (**Figure 2A, Supplemental Figure S1**). This observation showed that the growth
141 restoration of *ccr1-6* is not due to UGT72E family loss of function, but suggested that the
142 *ccr1-6* locus was epigenetically modified after crossing the *ugt72e1,-e2,-e3* and *ccr1-6*
143 mutants. Therefore, we renamed the growth-restored *ugt72e1,-e2,-e3 ccr1-6* and *ccr1-6*
144 mutants '*ugt72e1,-e2,-e3 epiccr1-6*' and '*epiccr1-6*', respectively, from here on.
145 Subsequently, we measured the biomass yield. As compared with the wild type, the length
146 and mass of the primary inflorescence stem, seed mass and total aerial plant biomass of
147 *ccr1-6* were reduced by 47%, 57%, 74%, and 55%, respectively (**Supplemental Table S1**).
148 In contrast, the biomass yield of *ugt72e1,-e2,-e3 epiccr1-6* mutants and *epiccr1-6* mutants
149 were similar to those of wild-type plants (**Figure 2A, Supplemental Table S1**).

150 **The *CCR1* expression and lignin content are partially restored in *ugt72e1,-e2,-e3*** 151 ***epiccr1-6* and *epiccr1-6* mutants**

152 To examine whether the observed biomass yield restoration in *ugt72e1,-e2,-e3 epiccr1-6* and
153 *epiccr1-6* mutants is due to relignification, we determined their lignin content. For lignin
154 content analysis, inflorescence stem cross sections were stained with the Wiesner or Mäule
155 reagent. Wiesner staining is used as a method to visualize coniferaldehyde residues in lignin.
156 Consistent with previous analyses (De Meester et al., 2018), the Wiesner staining of *ccr1-6*
157 cross sections was less intense as compared with those of the wild type (**Figure 2B**),
158 implying that *ccr1-6* mutants have dramatically reduced lignin levels. The staining intensity of
159 xylem of *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* sections resembled that of the wild type,
160 whereas that of the interfascicular fibers was lighter than that of the wild type (**Figure 2B**).
161 Cross sections of *ccr1-6* also revealed the typical irregularly shaped and collapsed vessels
162 (Jones et al., 2001; De Meester et al., 2018), whereas those of *ugt72e1,-e2,-e3 epiccr1-6*
163 and *epiccr1-6* mutants were large and open, similar as the wild type. Mäule staining
164 visualizes S-unit rich lignin. With Mäule, interfascicular fibers stained brighter red than xylem
165 in wild-type cross sections, whereas no red staining was observed in *ccr1-6*. The staining
166 intensity of xylem of *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* cross sections resembled that
167 of the wild type, but the red coloration of the interfascicular fibers appeared to be less dark
168 than that of the wild type (**Figure 2B**). Together, these complementary staining techniques
169 suggested that xylem lignification in *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* mutant
170 inflorescence stems is similar to that in the wild type, but their lignin content is lower or their
171 lignin composition is changed in interfascicular fibers.

172 To quantify lignin, a crude cell wall residue (CWR) was first prepared by removing
173 solubles from the senesced inflorescence stem biomass (**Table 1, Supplemental Figure**
174 **S2**). The wild-type plants had 79.0% CWR, while *ccr1-6* mutants had only 71.3% CWR (i.e.,
175 a relative reduction of 10%), indicating that *ccr1-6* biomass has more solubles. In contrast,
176 the CWR of *epiccr1-6* (on average 77.0%) was higher than that of *ccr1-6* (i.e., a relative
177 increase of 7%) and not significantly different from that of the wild type. Next, the fraction of
178 lignin in these prepared CWRs was measured via the acetyl bromide method (**Table 1,**
179 **Supplemental Figure S2**). Wild-type CWR had 14.3% lignin, whereas *ccr1-6* mutants had
180 9% lignin (a relative reduction of 37%). The lignin amounts of *ugt72e1,-e2,-e3 epiccr1-6* and
181 *epiccr1-6* (on average 10.6%) were recovered to levels higher than those of *ccr1-6* (i.e., a
182 relative increase of 18%), but were still lower than those of the wild type (i.e., a relative
183 decrease of 26%). Thus, although phenotypically indistinguishable, the lignin content of
184 *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* was not completely restored to wild-type levels.

185 Finally, we measured *CCR1* expression levels. Correctly spliced *CCR1* transcript levels
186 in *ccr1-6* inflorescence stems were 4% of those in the wild type (**Figure 2C**). On the contrary,
187 correctly spliced *CCR1* transcript levels in *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* mutants
188 were on average 34% of the wild-type levels. Note that the *ccr1-6* mutants and the *ugt72e1,-*
189 *e2,-e3 epiccr1-6* and *epiccr1-6* mutants were homozygous for the T-DNA insertion at the
190 *CCR1* locus (**Supplemental Figure S1B**). We concluded that the T-DNA-containing intron
191 can be spliced out correctly, but that correct splicing occurs less efficiently for the *ccr1-6*
192 allele than for the *epiccr1-6* allele. Increased correctly spliced *CCR1* transcript levels, and not
193 UGT72E family loss of function, must be the cause of the elevated lignin levels and, hence,
194 the growth restoration observed in *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* mutants.

195 **Knocking out UGT72E1,-E2 and -E3 in ccr1-CRISPR is insufficient to enhance the** 196 **incorporation of FA in lignin and restore growth**

197 *CCR1* transcript levels were partially restored in *ugt72e1,-e2,-e3 epiccr1-6* mutants, making
198 them unsuitable to investigate our initial hypothesis that blocking 4-O-glucosylation of FA
199 could result in higher levels of FA in lignin and as such restore the *ccr1-6* growth phenotype.
200 Thus, to re-evaluate our hypothesis, we generated a full knockout of *ccr1* in the wild-type and
201 *ugt72e1,-e2,-e3* mutant backgrounds through CRISPR-Cas9 gene editing (**Supplemental**
202 **Figure S3**). The two independent *ccr1*-CRISPR single mutant lines were severely dwarfed at
203 mature stage (on average 25.5 cm versus 59.0 cm for the wild type), as well as the two
204 independent *ugt72e1,-e2,-e3 ccr1*-CRISPR mutant lines (on average 31.3 cm), although they
205 were significantly taller (on average 5.8 cm, i.e., an increase of 23%) as compared with the
206 *ccr1*-CRISPR mutants (**Figure 3, Supplemental Table S2**). However, the observed growth

207 restoration of *ugt72e1,-e2,-e3 ccr1*-CRISPR mutants was very small; the length and the
208 biomass of the primary inflorescence stems were, respectively, on average 47% shorter and
209 52% lighter than those of the wild type (**Supplemental Table S2**).

210 Next, we measured the lignin amount and composition in CWRs of *ccr1*-CRISPR and
211 *ugt72e1,-e2,-e3 ccr1*-CRISPR mutants. The proportion of CWR of *ccr1*-CRISPR line 1 and
212 both *ugt72e1,-e2,-e3 ccr1*-CRISPR lines was similar (on average 74%) and significantly
213 lower than that of the wild type (79%, **Supplemental Table S3**). Also the lignin amount in the
214 primary inflorescence stem of both *ccr1*-CRISPR and both *ugt72e1,-e2,-e3 ccr1*-CRISPR
215 lines was similar (on average 8%) and significantly less than that of the wild type (13%,
216 **Supplemental Table S3**). The minor growth restoration of *ugt72e1,-e2,-e3 ccr1*-CRISPR
217 mutants compared with *ccr1*-CRISPR could thus not be attributed to an increase in total
218 lignin amount.

219 Finally, we measured whether FA incorporation in lignin was increased in the *ugt72e1,-*
220 *e2,-e3 ccr1*-CRISPR mutants as compared with the *ccr1*-CRISPR mutants. The incorporation
221 of traditional monolignols and FA in lignin was quantified via the thioacidolysis reaction,
222 which releases three FA-derived units, two of which are linked via conventional β -O-4-
223 linkages (β -O-4-FA-I and β -O-4-FA-II) and the third derived from the bis- β -O-4-coupling of FA
224 (bis- β -O-4-FA) in lignin (Ralph et al., 2008). In accordance with previously published results
225 of Arabidopsis *CCR1*-deficient plants (Mir Derikvand et al., 2008; Van Acker et al., 2013; De
226 Meester et al., 2018), the released amounts of β -O-4-FA-I, β -O-4-FA-II and bis- β -O-4-FA
227 units increased on average about 7, 6 and 8 times, respectively, in *ccr1*-CRISPR mutants
228 compared with the wild type (**Table 2, Supplemental Figure S4, Supplemental Table S3**).
229 However, the relative abundances of each of the three FA-derived thioacidolysis units did not
230 increase in the *ugt72e1,-e2,-e3 ccr1*-CRISPR mutants compared with the *ccr1*-CRISPR
231 mutants. Altogether, these results disprove our initial hypothesis that knocking out the
232 *UGT72E* gene family in *ccr1* mutants could result in enhanced FA incorporation in the lignin
233 polymer and growth restoration.

234 **The T-DNA copies at the *CCR1* locus are hypomethylated in *ccr1-6* mutants and** 235 **hypermethylated in *ugt72e1,-e2,-e3 epiccr1-6* mutants**

236 To further investigate the causal mechanism of growth recovery in *epiccr1-6*, we examined
237 the genome and epigenome sequences of the T-DNA and dSpm element loci in the
238 *ugt72e1,-e2,-e3* and *ccr1-6* parental lines and in the resulting *ugt72e1,-e2,-e3 epiccr1-6*
239 mutant. For sequencing, we used the Oxford Nanopore Technologies (ONT) platform. First,
240 we found that all mutants carried additional unanticipated T-DNA insertions (**Supplemental**
241 **Figure S5**). Second, we learned that for SAIL and GABI-Kat, T-DNA insertions occurred not

242 as just one single copy, but as a concatemer of (stretches of) direct and inverse repeats of
243 the T-DNA sequence that often also contained (part of) the vector backbone sequence
244 (**Supplemental Figure S5**).

245 Because the growth restoration observed in *ugt72e1,-e2,-e3 epiccr1-6* mutants is not
246 caused by UGT72E family loss of function (as proven by the *epiccr1-6* and *ugt72e1,-e2,-e3*
247 *ccr1*-CRISPR mutant phenotypes), we hypothesized that the epigenomic landscape of *ccr1-6*
248 mutants was altered after the genetic cross of *ccr1-6* and *ugt72e1,-e2,-e3* mutant
249 backgrounds. In plants, 5-methylcytosine in the CG dinucleotide context is a prominent
250 epigenetic DNA modification. Because ONT technology directly sequences genomic DNA,
251 i.e., without DNA amplification steps, DNA methylation is maintained and can be determined.
252 We found that the T-DNAs integrated at the *CCR1* locus were differentially methylated in
253 *ccr1-6* and *ugt72e1,-e2,-e3 epiccr1-6* mutant backgrounds. Only a part near the right border
254 of the T-DNA (a portion of the *pUC18* vector and the 35S promoter) of the *ccr1-6* allele was
255 hypermethylated, whereas almost the entire T-DNA of the *epiccr1-6* allele was
256 hypermethylated in the *ugt72e1,-e2,-e3 epiccr1-6* mutant (**Figure 4, A and B**). Only the
257 sulfonamide resistance gene on the T-DNAs, despite the presence of numerous CG
258 dinucleotides, was less methylated in the *epiccr1-6* allele as compared with the other regions
259 of the T-DNA (**Figure 4B**). In conclusion, the differential methylation status of the *ccr1-6* and
260 *epiccr1-6* alleles is most likely the cause of the observed enhanced splicing efficiency of
261 *CCR1* in the *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* mutants, and thus also the cause of
262 the observed growth restoration. Based on the restored growth phenotype of the F3, F4 and
263 F5 generation of *epiccr1-6*, the methylation status of the *epiccr1-6* allele seems to be
264 preserved over these subsequent generations.

265 In addition, we analyzed the methylation status of the other mutated loci (i.e., T-DNAs,
266 T-DNA flanking sequences and vector backbone-derived fragments) in the different mutant
267 backgrounds (**Supplemental Figure S6, 7 and 8**). The GABI-Kat T-DNAs in the *UGT72E1*
268 and AT3G51430 loci were partially methylated in the *ugt72e1,-e2,-e3* mutant (**Supplemental**
269 **Figure S6, C and D**). The mannopine synthase 1' 2' promoter, *pUC18* vector portion,
270 ampicillin resistance gene and 35S promoter of the GABI-Kat T-DNA carried dense
271 methylation, whereas the sulfonamide resistance gene was barely methylated. The
272 methylation degree of these regions was increased in the *ugt72e1,-e2,-e3 epiccr1-6* mutant,
273 especially in the left T-DNA region flanking the mannopine synthase 1' 2' promoter
274 (**Figure 4C, Supplemental Figure S8D**). Notably, the methylation degree of these GABI-Kat
275 T-DNAs was higher than that of the *ccr1-6* allele, but lower than that of the *epiccr1-6* allele.
276 In *ccr1-6* mutants, the GABI-Kat T-DNAs in AT1G77990 showed a methylation pattern
277 similar to that of the *ccr1-6* allele (**Figure 4A, Supplemental Figure S7B**). The SM dSpm
278 element in the *UGT72E2* locus was not methylated in the *ugt72e1,-e2,-e3* background

279 (Supplemental Figure S46H) and remained as such in the *ugt72e1,-e2,-e3 epiccr1-6*
280 background (Figure 4D). Copies of the SAIL T-DNA in the *UGT72E3* and AT5G35840 loci in
281 the *ugt72e1,-e2,-e3* mutant were hypermethylated, with the exception of the BASTA
282 resistance gene (Supplemental Figure S6, E and F). The methylation status of the SAIL T-
283 DNAs in *UGT72E3* in the *ugt72e1,-e2,-e3 epiccr1-6* mutant was similar as that in the
284 *ugt72e1,-e2,-e3* mutant, i.e., hypermethylated (Figure 4E). In short, we found that the T-
285 DNAs integrated in *CCR1*, *UGT72E1* and *UGT72E3*, and not the dSpm element in
286 *UGT72E2*, were already methylated before crossing and that the methylation was preserved,
287 or increased after crossing. We also found that methylation did not spread into the T-DNA
288 flanking sequences. A notable exception could be the GABI-Kat T-DNA inserted in the
289 AT1G77990 locus in the *ccr1-6* mutant background, where methylation was observed in one
290 of the flanking sequences (Supplemental Figure S7B). However, because this intronic
291 region was also partially methylated in the *ugt72e1,-e2,-e3* (Supplemental Figure S6B) and
292 *ugt72e1,-e2,-e3 epiccr1-6* (Supplemental Figure S8B) mutants, it is doubtful that the T-DNA
293 insertion triggered the methylation of this particular T-DNA flanking sequence. Finally, the
294 GABI-Kat (Figure 4, A and B) but not the SAIL (Figure 4E, Supplemental Figure S6E)
295 vector backbone-derived sequences were more heavily methylated in the *ugt72e1,-e2,-e3*
296 *epiccr1-6* background (after *trans* T-DNA suppression) compared with the *ugt72e1,-e2,-e3*
297 and *ccr1-6* backgrounds (before *trans* T-DNA suppression).

298 **The *ugt72e3* allele triggers *trans* T-DNA suppression of the *ccr1-6* allele**

299 To find out which of the *ugt72e* T-DNA mutant alleles is the trigger of *trans* T-DNA
300 suppression of the *ccr1-6* allele, we crossed each of the individual *ugt72e1*, *ugt72e2* and
301 *ugt72e3* T-DNA insertion mutants with the *ccr1-6* mutant. All plants homozygous for the *ccr1-*
302 *6* allele in the F2 progeny of the reciprocal crosses of *ugt72e1* x *ccr1-6* (7 out of 47 plants;
303 Supplemental Figure S9, A and B) and *ugt72e2* x *ccr1-6* (12 out of 72 plants;
304 Supplemental Figure S9, C and D) were dwarfed. In contrast, all F2 progeny plants of the
305 reciprocal crosses of *ugt72e3* x *ccr1-6* that were homozygous for the *ccr1-6* allele had a
306 restored growth phenotype (7 out of 47 plants; Supplemental Figure S9, E and F). We
307 concluded that the *ugt72e3* allele suppressed the *ccr1-6* mutant dwarfed phenotype in both
308 the *ccr1-6* x *ugt72e3* and *ccr1-6* x *ugt72e1,-e2,-e3* cross.

309 To date, only five cases have been reported in which the gene function of a T-DNA
310 insertion mutant was restored after a genetic cross with another T-DNA mutant
311 (Supplemental Table S4). The phenomenon is termed *trans* T-DNA suppression and the
312 minimal requirements for it to occur have been described as: (1) crossing two mutants
313 sharing homologous T-DNAs (T-DNA lines from the same mutant collection) of which (2) the

314 suppressed T-DNA is inserted into an intronic region (Osabe et al., 2017). The suppressed
315 T-DNA insertion in the *epiccr1-6* allele fulfills the second requirement: *ccr1-6* has an intronic
316 T-DNA insertion. However, the trigger T-DNA in *ugt72e3* (SAIL) and the suppressed T-DNA
317 in *ccr1-6* (GABI-Kat) originate from a different T-DNA collection and thus conflict with the first
318 requirement. Nonetheless, we found that among all pairwise sequence alignments of T-DNA
319 (and dSpm element) sequences of the most commonly used T-DNA consortia (and
320 transposant) collections, the T-DNA sequences of the SAIL and GABI-Kat collections share
321 the most sequence homology (**Figure 5**). The T-DNA sequences of *pDAP101* (SAIL) and
322 *pAC161* (GABI-Kat) share, in sense orientation, the mannopine synthase 1' 2' promoter (484
323 bp; Velten et al., 1984; Ülker et al., 2008) and 35S terminator (233 bp) sequences. In
324 antisense orientation, they share part of the *pUC18* vector sequence encoding among others
325 ampicillin resistance (2,161 bp), and an additional vector sequence (158 bp). It thus seems
326 that also one or more of these shorter stretches of identical sequences can trigger *trans* T-
327 DNA suppression.

328 **Prevalence of *trans* T-DNA suppression**

329 To estimate the prevalence of *trans* T-DNA suppression in Arabidopsis research, we
330 performed a literature search for the query 'T-DNA double mutant' (**Supplemental**
331 **Table S5**). We focused on articles published between 2015 and August 2022, because,
332 previously, a similar search was done for articles published in the period from 2002 till 2015
333 (Jia et al., 2015). From the 111 publications that matched our query, 24 (22%) reported
334 double or higher-order T-DNA mutants that met the requirements for *trans* T-DNA
335 suppression. We concluded that, since the first report of suppression of an intronic T-DNA
336 mutant (Xue et al., 2012), many researchers still made double or higher-order mutants
337 without considering the possibility of *trans* T-DNA suppression of intronic T-DNA mutants.
338 Only two publications reported the suppression of the mutant phenotype in the double or
339 higher-order mutant. The first paper describes the instability of the Arabidopsis *csn5a-2*
340 mutant and proved this was indeed a case of *trans* T-DNA suppression (Jia et al., 2015). The
341 second paper showed that the strong reduction of shoot growth associated with the severe
342 phosphate deficiency of the *pho1-7* T-DNA mutant was alleviated in the T-DNA *tex1 pho1-7*
343 double mutant as a consequence of the generation of a near full-length *PHO1* mRNA by
344 read-through transcription and splicing out of the T-DNA-containing intron (Khan et al.,
345 2020). Based on these observations, a role for the THO/TREX complex, of which TEX1 is a
346 component, in transcription termination was proposed. However, one can argue that the
347 growth restoration of *pho1-7* is not caused by the THO/TREX transcription termination
348 complex, but merely resulted from *trans* T-DNA suppression of *pho1-7* intronic T-DNA.

349 Notably, the relatively low prevalence of reports on *trans* T-DNA suppression might be an
350 underestimation, because the observations that follow from this suppression phenomenon
351 might have been considered as ‘aberrant’ or ‘out of scope’ for research teams, and might
352 therefore not have been reported.

353 Furthermore, we also screened all publications reporting a (partial) restoration of
354 mutant phenotypes in our research field, i.e., in cell-wall engineering (**Supplemental**
355 **Table S6**). A previous publication from our group reported the complete growth restoration of
356 *ccr1-6* via reintroduction of the *CCR1*-encoding sequence under control of the artificial
357 *SECONDARY NAC BINDING ELEMENT (SNBE)* promoter (*proSNBE*) that solely confers
358 expression in the vessels (De Meester et al., 2018). Both the GABI-Kat T-DNA and the
359 *proSNBE:CCR1* T-DNA contain the CaMV 35S minimal promoter sequence (46 bp). To
360 investigate whether the growth restoration observed after the transformation of the *ccr1-6*
361 mutant with the *proSNBE:CCR1* construct is caused by the generation of an *epiccr1-6*, we
362 transformed *ccr1*-CRISPR line 2 with the *proSNBE:CCR1* construct. Because the *ccr1*-
363 CRISPR line does not contain a T-DNA, the growth restoration observed in *ccr1*-CRISPR
364 *proSNBE:CCR1* cannot be caused by *trans* T-DNA suppression and must thus be solely
365 caused by the *proSNBE*-driven *CCR1* expression. The growth phenotype and length of the
366 primary inflorescence stem of the T3 single-locus homozygous *ccr1*-CRISPR
367 *proSNBE:CCR1* plants were similar to those of *ccr1-6 ProSNBE:CCR1* and wild-type plants
368 (**Supplemental Figure S10, Supplemental Table S7**), thus, also the growth restoration of
369 *ccr1-6 ProSNBE:CCR1* mutants is most likely not a case of *trans* T-DNA suppression. Its
370 mass was not completely restored to wild-type levels, although this was the case for *ccr1-6*
371 *proSNBE:CCR1* mutants (**Supplemental Table S7**). A position effect of the *proSNBE:CCR1*
372 construct might explain this minor discrepancy. Alternatively, the residual *CCR1* expression
373 measured in *ccr1-6* mutants might account for this difference. In the meantime, vessel-
374 specific relignification has independently been confirmed to be a valid strategy to fully restore
375 growth of Arabidopsis *ccr1*-CRISPR mutants (Yu et al., 2021). For none of the other 14
376 reports of (partial) growth restoration, we did find evidence that *trans* T-DNA suppression
377 would have caused the observed phenotypes.

378 Discussion

379 Multiple T-DNA insertion loci, each consisting of several T-DNA copies, and a deletion 380 as a consequence of T-DNA insertion mutagenesis

381 Initially, the objective of this research was to overcome lignin-modification-induced dwarfism
382 of Arabidopsis *ccr1* mutants by reducing the glucosylation of FA in order to enhance its

383 incorporation in lignin. We found that knocking out *UGT72E1,-E2* and *-E3* is insufficient to
384 complement the *ccr1* dwarfed phenotype and, instead, investigated the cause of the
385 complete growth restoration observed in *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* T-DNA
386 mutants.

387 From the whole-genome ONT sequencing performed to study the methylation pattern
388 of the T-DNA loci, we also obtained a detailed picture of the number of T-DNA inserts and
389 their organization. In addition to the anticipated insertions, we found additional T-DNA
390 insertions in the *ugt72e1,-e2,-e3*, *ccr1-6* and *ugt72e1,-e2,-e3 epiccr1-6* mutants. The
391 integration of T-DNAs at multiple sites in the genome is a well-known phenomenon. The
392 number of loci with T-DNA insertions per line is estimated to be on average 1.3 – 1.9 for
393 GABI-Kat (Rosso et al., 2003; Pucker et al., 2021), and 1.5 – 2.0 for SAIL (McElver et al.,
394 2001). In line with the expectations, the dSpm transposon element appeared as a single
395 insert in the *ugt72e2* locus only (Tissier et al., 1999). In *ugt72e1,-e2,-e3*, additional SAIL T-
396 DNAs were inserted in AT5G35840, encoding the apoprotein of phytochrome C (phyC).
397 PhyC plays a role in the perception of daylight; *phyC* mutants flower early when grown in
398 short-day photoperiods (Monte et al., 2003). Early flowering was also observed for the
399 *ugt72e3* SAIL T-DNA mutant (Baldacci-Cresp et al., 2020) and the subsequent proposed
400 relationship between *UGT72E3* and flowering is now questionable. We also found that three
401 protein-encoding genes neighboring the *UGT72E3* gene were deleted. Recently, a role for
402 *UGT72E3* in lignification of secondary cell walls was proposed based on effects observed in
403 the cell wall and transcriptome of *ugt72e3* (Baldacci-Cresp et al., 2020). Knowledge about
404 the three deleted genes neighboring *ugt72e3* is incomplete, but so far these could not be
405 linked with lignin biosynthesis nor growth, but we cannot exclude they do so in a *ccr1* mutant
406 background.

407 Moreover, we found that several T-DNA insertion sites consist of concatemers of direct
408 and inverse repeats of the T-DNA and even contain vector backbone sequence. These data
409 are in line with recent studies of other Arabidopsis T-DNA mutants of the SALK, SAIL, and
410 WISC collection (Jupe et al., 2019) and the GABI-Kat collection (Pucker et al., 2021). In
411 addition, these recent studies also report large-scale genomic rearrangements. We did not
412 observe the latter in the *ugt72e1,-e2,-e3*, *ccr1-6* and *ugt72e1,-e2,-e3 epiccr1-6* mutants.
413 Nonetheless, we showed that the genomic backgrounds of the T-DNA mutants used for this
414 analysis were more complex than initially anticipated.

415 **Differential methylation of T-DNA copies before and after crossing**

416 Nanopore sequencing-based methylation calling is the most recently developed technique to
417 study the methylome (Gigante et al., 2019; Gouil and Keniry, 2019). In the *ccr1-6* mutant

418 background, only the right part of every GABI-Kat T-DNA copy of the *ccr1-6* allele was
419 methylated, whereas after crossing, in the *ugt72e1,-e2,-e3 epiccr1-6* mutant background, we
420 observed dense methylation throughout the entire GABI-Kat T-DNA copies of the
421 suppressed *epiccr1-6* allele. Methylation of T-DNA insertions has been previously described.
422 One study determined the methylation pattern of the T-DNA insertions of a SALK and a SAIL
423 line via bisulfite sequencing (Jupe et al., 2019). They found an enrichment of methylation of
424 the entire T-DNA region in the SALK line and only the reporter gene – and not the resistance
425 gene – in the SAIL line. The fact that the methylation pattern of a T-DNA can change is in
426 line with the findings of another study in which the DNA methylation status of two SALK T-
427 DNA insertion loci were determined via bisulfite sequencing before and after crossing their
428 respective genotypes (Osabe et al., 2017). Large portions of the T-DNA sequences were
429 found to be already heavily methylated before crossing, and from the F1 generation of the
430 reciprocal cross onward, methylation was induced further and covered the entire T-DNA
431 sequence after crossing. Similarly to the phenotype of *ugt72e1,-e2,-e3 epiccr1-6* mutants,
432 the mutant phenotype of the hypermethylated intronic T-DNA insertion mutation was
433 alleviated after the cross (Osabe et al., 2017).

434 **Refining the existing and adding new requirements for *trans* T-DNA suppression**

435 The complete growth restoration of *ccr1* dwarfism observed in *ugt72e1,-e2,-e3 epiccr1-6* and
436 *epiccr1-6* mutants is caused by *trans* T-DNA suppression, an epigenetic phenomenon in
437 which gene function is restored after a genetic cross of T-DNA mutants (Xue et al., 2012;
438 Gao and Zhao, 2013; Sandhu et al., 2013; Jia et al., 2015; Osabe et al., 2017). The
439 molecular mechanism of *trans* T-DNA suppression is not yet fully understood, but a model for
440 the initiation and maintenance was proposed (Osabe et al., 2017). The initiation is similar to
441 the suppression of transposable elements in transcriptionally active regions, a natural plant
442 genome surveillance hack (Fultz et al., 2015; Saze, 2018). Our case of *trans* T-DNA
443 suppression of the *epiccr1-6* allele after a genetic cross of *ugt72e3* and *ccr1-6* T-DNA
444 mutants supports the proposed model for the initiation (**Figure 6**). Maintenance of *trans* T-
445 DNA suppression requires a heterochromatic state, maintained by methyltransferase 1
446 (MET1), a CG dinucleotide-specific DNA methyltransferase, and decreased DNA methylation
447 1 (DDM1), a chromatin remodeller (Osabe et al., 2017). As such, at the RNA level, both
448 intron splice sites are brought in physical proximity and splicing of the T-DNA-containing
449 intron is promoted by increased in bonsai methylation 2 (IBM2) and enhanced downy mildew
450 2 (EDM2) that recognize heterochromatin and enable splicing (Osabe et al., 2017).

451 To date, two requirements for *trans* T-DNA suppression to occur are defined (Osabe et
452 al., 2017; see above). However, our observation that the combination of T-DNAs of different

453 T-DNA collections also enables *trans* T-DNA suppression, instigates us to rephrase the first
454 requirement as follows: the presence of two T-DNAs sharing stretches of identical sequence.
455 To help researchers circumvent *trans* T-DNA suppression when creating a double or higher-
456 order T-DNA mutant, we made the pairwise sequence alignment of all T-DNA sequences
457 (and dSpm elements) of the most commonly used T-DNA mutant (and transposant)
458 collections (**Figure 5**). All combinations showed some degree of sequence homology,
459 requiring caution for *trans* T-DNA suppression any time an intronic T-DNA mutant is involved.
460 A notable exception is the combination of GABI-Kat T-DNA with a JIC SM transposon line, as
461 the respective sequences show basically no homology. Finally, we like to stress that not only
462 the act of crossing T-DNA mutants but also adding multiple T-DNA sequences to the plant
463 genome via transformation might result in *trans* T-DNA suppression of intronic T-DNA
464 mutants. The second requirement: the suppressed T-DNA is inserted into an intronic region,
465 remains intact.

466 The low prevalence of cases of *trans* T-DNA suppression in literature in combination
467 with the absence of *trans* T-DNA suppression in the *ccr1-6 ugt72e1* mutant (where both *ccr1-*
468 *6* and *ugt72e1* are GABI-Kat lines) shows that, even if the minimum requirements for *trans* T-
469 DNA suppression are met, suppression is not necessarily triggered. Thus, in addition to the
470 first two prerequisites, we propose two extra requirements for *trans* T-DNA suppression. Both
471 additional requirements would be needed to trigger the initiation of T-DNA methylation, which
472 is a key factor in T-DNA suppression. The third requirement is the presence of direct T-DNA-
473 derived and reversed complement T-DNA-derived transcripts to enable the formation of
474 dsRNA, implying that the T-DNA-containing genes should be simultaneously expressed in
475 space and time. Both the T-DNA integration loci of *ugt72e3* and *ccr1-6* consist of direct and
476 inverse repeats of their T-DNA sequences. After the genetic cross of *ugt72e3* with *ccr1-6* and
477 *ugt72e1, -e2, -e3* with *ccr1-6*, primary transcripts (i.e., pre-mRNA) of both genes still contain
478 the T-DNA sequences and can form dsRNA (**Figure 6**). This condition was also met in the
479 study of Osabe et al. (2017). Unfortunately, the other reported cases of *trans* T-DNA
480 suppression can currently not be inspected for this prerequisite because of the lack of
481 genome sequence data. Notably, *ccr1-6* and *ccr1-6 ugt72e1* mutant backgrounds also had
482 the ability to form T-DNA-derived dsRNA, but *trans* T-DNA suppression was not triggered.
483 This notable exception lead us to speculate on the fourth requirement: the amount of T-DNA-
484 derived dsRNA needs to exceed a threshold value to trigger the initiation of T-DNA
485 methylation. Only after introducing copies of the T-DNA sequence that are also sufficiently
486 transcribed (here: T-DNA copies in the *ugt72e3* allele) into the *ccr1-6* mutant background,
487 the amount of T-DNA-derived dsRNA surpassed the threshold value to trigger methylation of
488 the T-DNA in the *ccr1-6* locus.

489 In conclusion, we found that *trans* T-DNA suppression was at the origin of the growth
490 recovery of the *ugt72e1,-e2,-e3 epiccr1-6* and *epiccr1-6* mutants. We reach out to the
491 Arabidopsis research community to consider the possibility of *trans* T-DNA suppression
492 when interpreting newly generated or reported data of intronic T-DNA mutants, especially
493 when two (or more) T-DNA mutants are combined.

494

495 **Materials and methods**

496 For a detailed description, see **Supplemental Materials and Methods**. In brief, the
497 *Arabidopsis ccr1-6* T-DNA mutant (GK_622C01) (Mir Derikvand et al., 2008) was crossed
498 with the *ugt72e1* (GK_340H02),-*e2* (SM_3_20654),-*e3* (SAIL_1279_D02) T-DNA mutant, of
499 which each of the individual *ugt72e* mutants was described previously (Baldacci-Cresp et al.,
500 2020). *Arabidopsis ccr1*-CRISPR knockout mutants in wild-type and *ugt72e1,-e2,-e3* mutant
501 background were generated with CRISPR-Cas9 gene editing technology. For cloning, we
502 adopted the protocol of Decaestecker et al. (2019) that makes use of the GreenGate cloning
503 toolbox (Lampropoulos et al., 2013). In the complementation study, the *ccr1*-CRISPR line 2
504 mutant was transformed with the *ProSNBE:CCR1* construct, in which *CCR1* is expressed
505 under a vessel-specific promoter, previously described by De Meester et al. (2018).

506 For the stem phenotype, biomass measurements and analysis of the lignin content and
507 composition, seeds were sown in soil and stratified at 4°C for 4 days prior to placing them
508 under long-day conditions (16-h light/8-h dark photoperiods). For microscopy, seeds were
509 sown in soil and stratified at 4°C for 4 days prior to placing them under short-day conditions
510 (8-h light/16-h dark photoperiods). After 5 weeks in short-day conditions, plants were
511 transferred to long-day conditions for 4 weeks and main stems were harvested.

512 Analysis of the lignin content was performed according to the acetyl bromide method as
513 essentially described by Dence (1992). The general lignin composition and the quantification
514 of FA-derived lignin units were determined using thioacidolysis as essentially described by
515 Robinson and Mansfield (2009) and modified by De Meester et al. (2018). Response factors
516 for H, G, S and FA-derived units were calculated according to Yue et al. (2012). For lignin
517 microscopy, stem sections were stained with Wiesner and Mäule reagents (Pradhan Mitra
518 and Loqué, 2014) and imaged with an Olympus BX51 microscope coupled to a Zeiss ICc1
519 camera with a Plan 10X 0.25 dry objective.

520 For transcript analysis, 1 rosette leaf of 3 individual 25-day-old plants were pooled.
521 Total RNA was isolated using the ReliaPrep™ RNA Tissue Miniprep System (Promega).
522 Total cDNA was synthesized using qScript® cDNA SuperMix (Quantabio). The relative
523 expression level was determined with the Roche LightCycler 480 combined with the SYBR
524 Green I Master Kit (Roche Diagnostics) in three technical repeats.

525 High-molecular weight genomic DNA was extracted from rosette leaves for whole-
526 genome sequencing. From the obtained gDNA, a library was constructed using the 1D
527 Sequencing Kit following the manufacturer's protocol (SQK-LSK109, ONT). Afterwards, the
528 library was loaded onto a MinION Flow cell (R9.4.1, ONT) and sequenced using a MinION
529 sequencing device (ONT). Base calling of the raw reads was done using guppy (version
530 5.0.7, <http://www.nanoporetech.com>). For each mutant, the ONT reads were assembled

531 using the Flye software (v2.8) (Kolmogorov et al., 2019) and each assembly was further
532 polished using Nanopolish (Hu et al., 2021). The obtained polished genomes were then
533 purged for haplotigs remaining in the assemblies (Roach et al., 2018). Contigs of the
534 individual *de novo* genome assemblies were ordered and oriented with the 'Ragoo' tool
535 (Alonge et al., 2019) using the reference Arabidopsis TAIR10 genome as a guide (Lamesch
536 et al., 2012). Genes were predicted on individual polished genome assemblies using protein-
537 coding genes from Araport11 (Cheng et al., 2017) and the Augustus gene prediction
538 software (Stanke et al., 2008). Raw ONT sequencing data (FAST5 format) was deposited in
539 the Sequence Read Archive (SRA) on NCBI under project PRJNA767312.

540 The methylome was obtained with Megalodon (v2.3.3, with guppy 5.0.7,
541 <https://github.com/nanoporetech/megalodon>) and visualization of the methylation patterns of
542 loci of interest was executed with the Methylartist tool (Cheetham et al., 2022). Complex T-
543 DNA insertion loci, including gene prediction models, as well as ideograms of the individual
544 Arabidopsis T-DNA mutant lines were plotted within RStudio using a custom script (R version
545 4.0.4). Some of those custom R plots were manually added to the upper track of the
546 Methylartist plot.

547 For the pairwise sequence alignments of the complete T-DNA (dSpm element)
548 sequences (**Supplemental Sequences**), the dotplot feature in Geneious Prime® 2022.0.1
549 (<https://www.geneious.com>) was used.

550 Statistical analyses were performed with RStudio, version 3.6.3.

551 **Supplemental data**

552 The following materials are available in the online version of this article.

553 **Supplemental Materials and methods**

554 **Supplemental Figure S1:** The *ccr1-6* mutants and the *ugt72e1,-e2,-e3 epiccr1-6* and
555 *epiccr1-6* mutants are homozygous for the T-DNA insertion at the *CCR1* locus.

556 **Supplemental Figure S2:** Dot and box plot representation of Table 1.

557 **Supplemental Figure S3:** CRISPR-induced *ccr1* mutations in wild-type and *ugt72e1,-e2,-*
558 *e3* mutant backgrounds.

559 **Supplemental Figure S4:** Dot and box plot representation of Table 2.

560 **Supplemental Figure S5:** Ideogram of the five chromosomes and zoom-in of the different
561 T-DNA and vector backbone arrays integrated in different Arabidopsis mutants.

562 **Supplemental Figure S6:** Methylation profile plot of different loci in the *ugt72e1,-e2,-e3*
563 mutant background.

564 **Supplemental Figure S7:** Methylation profile plot of different loci in the *ccr1-6* mutant
565 background.

566 **Supplemental Figure S8** Methylation profile plot of different loci in the *ugt72e1,-e2,-e3*
567 *epiccr1-6* mutant background.

568 **Supplemental Figure S9:** Phenotype of F2 progeny plants of the reciprocal crosses of
569 *ccr1-6* with either of the *ugt72e1/ugt72e2/ugt72e3* mutants.

570 **Supplemental Figure S10:** Growth restoration of the *ccr1*-CRISPR mutant phenotype by
571 stacking the *proSNBE:CCR1* construct and the *ccr1-6* mutation.

572 **Supplemental Table S1:** Biomass measurements of *ugt72e1,-e2,-e3 epiccr1-6* and
573 *epiccr1-6* mutants.

574 **Supplemental Table S2:** Biomass measurements of the *ccr1*-CRISPR and *ugt72e1,-e2,-*
575 *e3 ccr1*-CRISPR mutants.

576 **Supplemental Table S3:** Crude cell wall residue (CWR), lignin content and lignin
577 composition of *ccr1*-CRISPR and *ugt72e1,-e2,-e3 ccr1*-CRISPR mutants.

578 **Supplemental Table S4:** Confirmed cases of *trans* T-DNA suppression.

579 **Supplemental Table S5:** Prevalence of *trans* T-DNA suppression in literature.

580 **Supplemental Table S6:** Prevalance of *trans* T-DNA suppression in the cell-wall
581 engineering research field.

582 **Supplemental Table S7:** Biomass measurements of different *ccr1* mutant lines.

583 **Supplemental Table S8:** Primer sequences and primer pair-optimized annealing
584 temperature for PCR-based genotyping of different T-DNA insertion loci.

585 **Supplemental Table S9:** gRNA target sequences.

586 **Supplemental Table S10:** Oligonucleotide sequences used for cloning, vector validation
587 and genotyping of *ccr1*-CRISPR mutants.

588 **Supplemental Table S11:** Vector overview for Golden Gate cloning.

589 **Supplemental Table S12:** Summary of specific quantifier, qualifiers, retention time (RT)
590 and response factor (RF) used for GC-MS-based analysis of lignin monomers released
591 upon thioacidolysis.

592 **Supplemental Table S13:** Primer sequences for RT-qPCR.

593 **Supplemental Sequences**

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604 **Conflict of interest**

605 The authors declare that the research was conducted in the absence of any commercial or
606 financial relationships that could be construed as a potential conflict of interest.

607 **References**

- 608 **Alonge M, Soyk S, Ramakrishnan S, Wang X, Goodwin S, Sedlazeck FJ, Lippman ZB,**
609 **Schatz MC** (2019) RaGOO: fast and accurate reference-guided scaffolding of draft
610 genomes. *Genome Biol* **20**: 224
- 611 **Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK,**
612 **Zimmerman J, Barajas P, Cheuk R, et al** (2003) Genome-wide insertional
613 mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653-657
- 614 **Baldacci-Cresp F, Le Roy J, Huss B, Lion C, Créach A, Spriet C, Duponchel L, Biot C,**
615 **Baucher M, Hawkins S, et al** (2020) *UDP-GLYCOSYLTRANSFERASE 72E3* plays a
616 role in lignification of secondary cell walls in *Arabidopsis*. *Int J Mol Sci* **21**: 6094
- 617 **Bonawitz ND, Kim JI, Tobimatsu Y, Ciesielski PN, Anderson NA, Ximenes E, Maeda J,**
618 **Ralph J, Donohoe BS, Ladisch M, et al** (2014) Disruption of Mediator rescues the
619 stunted growth of a lignin-deficient *Arabidopsis* mutant. *Nature* **509**: 376–380
- 620 **Böhmdorfer G, Sethuraman S, Jordan Rowley M, Krzyszton M, Hafiz Rothi M, Bouzit L,**
621 **Wierzbicki AT** (2016) Long non-coding RNA produced by RNA polymerase V
622 determines boundaries of heterochromatin. *eLife* **5**: e19092.
- 623 **Bowles D, Lim E-K, Poppenberger B, Vaistij FE** (2006) Glycosyltransferases of lipophilic
624 small molecules. *Annu Rev Plant Biol* **57**: 567-597
- 625 **Chanoca A, de Vries L, Boerjan W** (2019) Lignin engineering in forest trees. *Front Plant Sci*
626 **10**: 912
- 627 **Cheetham SW, Kindlova M, Ewing AD** (2022) Methylartist: tools for visualising modified
628 bases from nanopore sequence data. *Bioinformatics* **38**: 3109-3112

- 629 **Chen F, Dixon RA** (2007) Lignin modification improves fermentable sugar yields for biofuel
630 production. *Nat Biotechnol* **25**: 759-761
- 631 **Cheng C-Y, Krishnakumar V, Chan AP, Thibaud-Nissen F, Schobel S, Town CD** (2017)
632 Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome.
633 *Plant J* **89**: 789-804
- 634 **De Meester B, de Vries L, Özparpucu M, Gierlinger N, Corneillie S, Pallidis A,**
635 **Goeminne G, Morreel K, De Bruyne M, De Rycke R, et al** (2018) Vessel-specific
636 reintroduction of CINNAMOYL-COA REDUCTASE1 (CCR1) in dwarfed *ccr1* mutants
637 restores vessel and xylary fiber integrity and increases biomass. *Plant Physiol* **176**:
638 611-633
- 639 **De Meester B, Madariaga Calderón B, de Vries L, Pollier J, Goeminne G, Van**
640 **Doorselaere J, Chen M, Ralph J, Vanholme R, Boerjan W** (2020) Tailoring poplar
641 lignin without yield penalty by combining a null and haploinsufficient *CINNAMOYL-*
642 *CoA REDUCTASE2* allele. *Nat Commun* **11**: 5020
- 643 **De Meester B, Vanholme R, de Vries L, Wouters M, Van Doorselaere J, Boerjan W**
644 (2021) Vessel-and ray-specific monolignol biosynthesis as an approach to engineer
645 fiber-hypolignification and enhanced saccharification in poplar. *Plant J* **108**: 752-765
- 646 **De Meester, B., Vanholme, R., Mota, T., and Boerjan, W.** (2022). Lignin engineering in
647 forest trees: from gene discovery to field trials. *Plant Commun* **3**:100465
- 648 **Decaestecker W, Andrade Bueno R, Pfeiffer ML, Vangheluwe N, Jourquin J, Karimi M,**
649 **Van Isterdael G, Beeckman T, Nowack MK, Jacobs TB** (2019) CRISPR-TSKO: a
650 technique for efficient mutagenesis in specific cell types, tissues, or organs in
651 *Arabidopsis*. *Plant Cell* **31**: 2868-2887
- 652 **Dence CW** (1992) The determination of lignin. *In* SY Lin, CW Dence, eds, *Methods in Lignin*
653 *Chemistry*. Springer-Verlag, Berlin, Germany, pp 33-61
- 654 **Dima O, Morreel K, Vanholme B, Kim H, Ralph J, Boerjan W** (2015) Small glycosylated
655 lignin oligomers are stored in *Arabidopsis* leaf vacuoles. *Plant Cell* **27**: 695-710
- 656 **Fultz D, Choudury SG, Slotkin RK** (2015) Silencing of active transposable elements in
657 plants. *Curr Opin Plant Biol* **27**: 67-76
- 658 **Gallego-Giraldo L, Liu C, Pose-Albacete S, Pattathil S, Peralta AG, Young J,**
659 **Westpheling J, Hahn MG, Rao X, Knox JP, et al** (2020) ARABIDOPSIS
660 DEHISCENCE ZONE POLYGALACTURONASE 1 (ADPG1) releases latent defense
661 signals in stems with reduced lignin content. *Proc Natl Acad Sci USA* **117**: 3281-3290
- 662 **Gao Y, Zhao Y** (2013) Epigenetic suppression of T-DNA insertion mutants in *Arabidopsis*.
663 *Mol Plant* **6**: 539-545
- 664 **Gigante S, Gouil Q, Lucattini A, Keniry A, Beck T, Tinning M, Gordon L, Woodruff C,**
665 **Speed TP, Blewitt ME, et al** (2019) Using long-read sequencing to detect imprinted
666 DNA methylation. *Nucleic Acids Res* **47**: e46
- 667 **Gouil Q, Keniry A** (2019) Latest techniques to study DNA methylation. *Essays Biochem* **63**:
668 639-648
- 669 **Hu K, Huang N, Zou Y, Liao X, Wang J** (2021) MultiNanopolish: refined grouping method
670 for reducing redundant calculations in Nanopolish. *Bioinformatics* **37**: 2757-2760
- 671 **Jia X, Chanda B, Zhao M, Brunner AM, Beers EP** (2015) Instability of the *Arabidopsis*
672 mutant *csn5a-2* caused by epigenetic modification of intronic T-DNA. *Plant Sci* **238**:
673 53-63
- 674 **Jones L, Ennos AR, Turner SR** (2001) Cloning and characterization of *irregular xylem4*
675 (*irx4*): a severely lignin-deficient mutant of *Arabidopsis*. *Plant J* **26**: 205-216

- 676 **Jupe F, Rivkin AC, Michael TP, Zander M, Motley ST, Sandoval JP, Slotkin RK, Chen H,**
677 **Castanon R, Nery JR, et al** (2019) The complex architecture and epigenomic impact
678 of plant T-DNA insertions. *PLoS Genet* **15**: e1007819
- 679 **Khan GA, Deforges J, Reis RS, Hsieh Y-F, Montpetit J, Antosz W, Santuari L, Hardtke**
680 **CS, Grasser KD, Poirier Y** (2020) The transcription and export complex THO/TREX
681 contributes to transcription termination in plants. *PLoS Genet* **16**: e1008732
- 682 **Kleinboelting N, Huet G, Kloetgen A, Viehoveer P, Weisshaar B** (2012) GABI-Kat
683 SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database.
684 *Nucleic Acids Res* **40**: D1211-D1215
- 685 **Kolmogorov M, Yuan J, Lin Y, Pevzner PA** (2019) Assembly of long, error-prone reads
686 using repeat graphs. *Nat Biotechnol* **37**: 540-546
- 687 **Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, Muller R, Dreher**
688 **K, Alexander DL, Garcia-Hernandez M, et al** (2012) The Arabidopsis Information
689 Resource (TAIR): improved gene annotation and new tools. *Nucleic Acids Res* **40**:
690 D1202-D1210
- 691 **Lampropoulos A, Sutikovic Z, Wenzl C, Maegele I, Lohmann JU, Forner J** (2013)
692 GreenGate - A novel, versatile, and efficient cloning system for plant transgenesis.
693 *PLoS ONE* **8**: e83043
- 694 **Lanot A, Hodge D, Jackson RG, George GL, Elias L, Lim E-K, Vaistij FE, Bowles DJ**
695 (2006) The glucosyltransferase UGT72E2 is responsible for monolignol 4-O-glucoside
696 production in *Arabidopsis thaliana*. *Plant J* **48**: 286-295
- 697 **Le Roy J, Huss B, Creach A, Hawkins S, Neutelings G** (2016) Glycosylation is a major
698 regulator of phenylpropanoid availability and biological activity in plants. *Front Plant*
699 *Sci* **7**: 735
- 700 **Lep le J-C, Dauwe R, Morreel K, Storme V, Lapierre C, Pollet B, Naumann A, Kang K-Y,**
701 **Kim H, Ruel K, et al** (2007) Downregulation of cinnamoyl-coenzyme A reductase in
702 poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and
703 structure. *Plant Cell* **19**: 3669-3691
- 704 **Lim E-K, Jackson RG, Bowles DJ** (2005) Identification and characterisation of *Arabidopsis*
705 glycosyltransferases capable of glucosylating coniferyl aldehyde and sinapyl
706 aldehyde. *FEBS Lett* **579**: 2802-2806
- 707 **Lim E-K, Li Y, Parr A, Jackson R, Ashford DA, Bowles DJ** (2001) Identification of
708 glucosyltransferase genes involved in sinapate metabolism and lignin synthesis in
709 *Arabidopsis*. *J Biol Chem* **276**: 4344-4349
- 710 **Matzke MA, Mosher RA** (2014) RNA-directed DNA methylation: an epigenetic pathway of
711 increasing complexity. *Nat Rev Genet* **15**: 394-408
- 712 **McElver J, Tzafrir I, Aux G, Rogers R, Ashby C, Smith K, Thomas C, Schetter A, Zhou**
713 **Q, Cushman MA, et al** (2001) Insertional mutagenesis of genes required for seed
714 development in *Arabidopsis thaliana*. *Genetics* **159**: 1751-1763
- 715 **Mir Derikvand M, Berrio Sierra J, Ruel K, Pollet B, Do C-T, Th venin J, Buffard D,**
716 **Jouanin L, Lapierre C** (2008) Redirection of the phenylpropanoid pathway to feruloyl
717 malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1. *Planta* **227**:
718 943-956
- 719 **Monte E, Alonso JM, Ecker JR, Zhang Y, Li X, Young J, Austin-Phillips S, Quail PH**
720 (2003) Isolation and characterization of *phyC* mutants in *Arabidopsis* reveals complex
721 crosstalk between phytochrome signaling pathways. *The Plant Cell* **15**: 1962-1980
- 722 **Muro-Villanueva F, Mao X, Chapple C** (2019) Linking phenylpropanoid metabolism, lignin
723 deposition, and plant growth inhibition. *Curr Opin Biotechnol* **56**: 202-208

- 724 **O'Malley RC, Barragan CC, Ecker JR** (2015) A user's guide to the Arabidopsis T-DNA
725 insertion mutant collections. *Methods Mol Biol* **1384**: 323-342
- 726 **Osabe K, Harukawa Y, Miura S, Saze H** (2017) Epigenetic regulation of intronic transgenes
727 in *Arabidopsis*. *Sci Rep* **7**: 45166
- 728 **Panda C, Li X, Wager A, Chen H-Y, Li X** (2020) An importin-beta-like protein mediates
729 lignin-modification-induced dwarfism in Arabidopsis. *Plant J* **102**: 1281-1293
- 730 **Pradhan Mitra P, Loqué D** (2014) Histochemical staining of *Arabidopsis thaliana* secondary
731 cell wall elements. *J Vis Exp* **87**: e51381
- 732 **Pucker B, Kleinbölting N, Weisshaar B** (2021) Large scale genomic rearrangements in
733 selected *Arabidopsis thaliana* T-DNA lines are caused by T-DNA insertion
734 mutagenesis. *BMC Genomics* **22**: 599
- 735 **Ralph J, Kim H, Lu F, Grabber JH, Leplé J-C, Berrio-Sierra J, Mir Derikvand M, Jouanin
736 L, Boerjan W, Lapierre C** (2008) Identification of the structure and origin of a
737 thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins
738 (and an indicator for cinnamoyl CoA reductase deficiency). *Plant J* **53**: 368-379
- 739 **Ralph J, Lapierre C, Boerjan W** (2019) Lignin structure and its engineering. *Curr Opin
740 Biotechnol* **56**: 240-249
- 741 **Roach MJ, Schmidt SA, Borneman AR** (2018) Purge Haplotigs: allelic contig reassignment
742 for third-gen diploid genome assemblies. *BMC Bioinformatics* **19**: 460
- 743 **Robinson AR, Mansfield SD** (2009) Rapid analysis of poplar lignin monomer composition
744 by a streamlined thioacidolysis procedure and near-infrared reflectance-based
745 prediction modeling. *Plant J* **58**: 706-714
- 746 **Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B** (2003) An *Arabidopsis
747 thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based
748 reverse genetics. *Plant Mol Biol* **53**: 247-259
- 749 **Sandhu KS, Koirala PS, Neff MM** (2013) The *ben1-1* brassinosteroid-catabolism mutation is
750 unstable due to epigenetic modifications of the intronic T-DNA insertion. *G3: Genes,
751 Genomes, Genet* **3**: 1587-1595
- 752 **Saze H** (2018) Epigenetic regulation of intragenic transposable elements: a two-edged
753 sword. *J Biochem* **164**: 323-328
- 754 **Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P,
755 Bacwaden J, Ko C, et al** (2002) A high-throughput Arabidopsis reverse genetics
756 system. *Plant Cell* **14**: 2985-2994
- 757 **Sibout R, Eudes A, Pollet B, Goujon T, Mila I, Granier F, Séguin A, Lapierre C, Jouanin
758 L** (2003) Expression pattern of two paralogs encoding cinnamyl alcohol
759 dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding
760 mutants. *Plant Physiol* **132**: 848-860
- 761 **Speckaert N, Adamou NM, Hassane HA, Baldacci-Cresp F, Mol A, Goeminne G,
762 Boerjan W, Duez P, Hawkins S, Neutelings G, et al** (2020) Characterization of the
763 UDP-glycosyltransferase UGT72 family in poplar and identification of genes involved
764 in the glycosylation of monolignols. *Int J Mol Sci* **21**: 5018
- 765 **Stanke M, Diekhans M, Baertsch R, Haussler D** (2008) Using native and syntenically
766 mapped cDNA alignments to improve *de novo* gene finding. *Bioinformatics* **24**: 637-
767 644
- 768 **Tissier AF, Marillonnet S, Klimyuk V, Patel K, Torres MA, Murphy G, Jones JDG** (1999)
769 Multiple independent defective *Suppressor-mutator* transposon insertions in
770 Arabidopsis: a tool for functional genomics. *Plant Cell* **11**: 1841-1852

- 771 **Ülker B, Peiter E, Dixon DP, Moffat C, Capper R, Bouché N, Edwards R, Sanders D,**
772 **Knight H, Knight MR** (2008) Getting the most out of publicly available T-DNA
773 insertion lines. *Plant J* **56**: 665-677
- 774 **Van Acker R, Vanholme R, Storme V, Mortimer JC, Dupree P, Boerjan W** (2013) Lignin
775 biosynthesis perturbations affect secondary cell wall composition and saccharification
776 yield in *Arabidopsis thaliana*. *Biotechnol Biofuels* **6**: 46
- 777 **Vanholme R, De Meester B, Ralph J, Boerjan W** (2019) Lignin biosynthesis and its
778 integration into metabolism. *Curr Opin Biotechnol* **56**: 230-239
- 779 **Vanholme R, Morreel K, Darrah C, Oyarce P, Grabber JH, Ralph J, Boerjan W** (2012a)
780 Metabolic engineering of novel lignin in biomass crops. *New Phytol* **196**: 978-1000
- 781 **Vanholme R, Storme V, Vanholme B, Sundin L, Christensen JH, Goeminne G, Halpin C,**
782 **Rohde A, Morreel K, Boerjan W** (2012b) A systems biology view of responses to
783 lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell* **24**: 3506-3529
- 784 **Velten J, Velten L, Hain R, Schell J** (1984) Isolation of a dual plant promoter fragment from
785 the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J* **3**: 2723-2730
- 786 **Vermaas JV, Dixon RA, Chen F, Mansfield SD, Boerjan W, Ralph J, Crowley MF,**
787 **Beckham GT** (2019) Passive membrane transport of lignin-related compounds. *Proc*
788 *Natl Acad Sci USA* **116**: 23117-23123
- 789 **Wambui Mbichi R, Wang Q-F, Wan T** (2020) RNA directed DNA methylation and seed plant
790 genome evolution. *Plant Cell Rep* **39**: 983-996
- 791 **Wang P, Guo L, Morgan J, Dudareva N, Chapple C** (2022) Transcript and metabolite
792 network perturbations in lignin biosynthetic mutants of *Arabidopsis*. *Plant Physiol*, in
793 press (10.1093/plphys/kiac344).
- 794 **Wu J, Zhu W, Shan X, Liu J, Zhao L, Zhao Q** (2022) Glycoside-specific metabolomics
795 combined with precursor isotopic labeling for characterizing plant
796 glycosyltransferases. *Mol Plant*, in press (10.1016/j.molp.2022.08.003).
- 797 **Xue W, Ruprecht C, Street N, Hematy K, Chang C, Frommer WB, Persson S, Niittyla T**
798 (2012) Paramutation-like interaction of T-DNA loci in *Arabidopsis*. *PLoS ONE* **7**:
799 e51651
- 800 **Yu H, Liu C, Dixon RA** (2021) A gene-editing/complementation strategy for tissue-specific
801 lignin reduction while preserving biomass yield. *Biotechnol Biofuels* **14**: 175
- 802 **Yue F, Lu F, Sun R-C, Ralph J** (2012) Syntheses of lignin-derived thioacidolysis monomers
803 and their uses as quantitation standards. *J Agric Food Chem* **60**: 922-928
- 804 **Zhai J, Bischof S, Wang H, Feng S, Lee T, Teng C, Chen X, Park SY, Liu L, Gallego-**
805 **Bartolome J, et al** (2015) One precursor One siRNA model for Pol IV-dependent
806 siRNAs Biogenesis. *Cell* **163**: 445-455

807 **Figure legends and tables**

808 **Figure 1.** The general phenylpropanoid and monolignol-specific pathways in *Arabidopsis*
809 wild type. Major fluxes are indicated by solid arrows, while minor fluxes are indicated by
810 dashed arrows. General phenylpropanoid pathway: PAL: phenylalanine ammonia-lyase;
811 C4H: cinnamate 4-hydroxylase; C3H: *p*-coumarate 3-hydroxylase; 4CL: 4-coumarate:CoA
812 ligase; HCT: hydroxycinnamoyl-CoA shikimate/quininate hydroxycinnamoyl transferase; C3'H:
813 *p*-coumaroyl shikimate 3'-hydroxylase; CSE: caffeoyl shikimate esterase; CCoAOMT:
814 caffeoyl-CoA *O*-methyltransferase. Monolignol-specific pathway: CCR: cinnamoyl-CoA
815 reductase (in blue); F5H: ferulate 5-hydroxylase; COMT: caffeic acid *O*-methyltransferase;

816 CAD: cinnamyl alcohol dehydrogenase. UGT72E: UDP-glycosyltransferase 72E (in red).
817 UGT84A: UDP-glycosyltransferase 84A. HCALDH: hydroxycinnamaldehyde dehydrogenase.
818 How feruloyl-CoA is converted into ferulic acid is currently unknown, it might be either
819 spontaneous or catalyzed via a thioesterase. H: *p*-hydroxyphenyl, G: guaiacyl, S: syringyl,
820 FA: ferulic acid.

821 **Figure 2.** The *ccr1-6* dwarfed phenotype is suppressed after a cross between *ccr1-6* and
822 *ugt72e1,-e2,-e3* mutants. A, Phenotype of seven-week-old *ugt72e1,-e2,-e3 epiccr1-6* and
823 *epiccr1-6* Arabidopsis plants grown under long-day conditions. The picture is representative
824 for multiple plants of each genotype/generation (n = 21, except for *epiccr1-6* F3: n = 3). Scale
825 bar = 5 cm. B, Tissue anatomy and lignin deposition in *ugt72e1,-e2,-e3 epiccr1-6* and
826 *epiccr1-6* mutants. Transverse stem sections of vascular bundles were stained with Wiesner
827 or Mäule reagent. V: vessel, XF: xylary fiber, IF: interfascicular fiber, P: phloem. Scale bars =
828 50 μ m. C, Relative *CCR1* expression levels in rosette leaves of 25-day-old *ugt72e1,-e2,-e3*
829 *epiccr1-6* and *epiccr1-6* mutants. The error bars designate the standard error of the mean (n
830 = 5). Values on the Y-axis represent *CCR1* transcript levels compared with the wild type (set
831 to 1). Different letters represent significant differences at the 0.05 significance level (ANOVA,
832 Tukey *post-hoc*).

833 **Figure 3.** Minor growth restoration of *ugt72e1,-e2,-e3 ccr1*-CRISPR mutants. Phenotype of
834 7-week-old Arabidopsis plants grown under long-day conditions. The picture is
835 representative for multiple plants of each genotype (n = 17). Scale bar = 5 cm.

836 **Figure 4.** Methylation profile plot of the T-DNA copies, dSpm element and vector backbone
837 sequences at different loci in the *ccr1-6* and *ugt72e1,-e2,-e3 epiccr1-6* mutant backgrounds.
838 A, *CCR1* locus in the *ccr1-6* mutant background. B, *CCR1* locus, C, *UGT72E1* locus, D,
839 *UGT72E2* locus and E, *UGT72E3* locus in the *ugt72e1,-e2,-e3 epiccr1-6* mutant background.
840 Windows of each plot were framed 1 kb upstream and 1 kb downstream of the gene
841 prediction of the *de novo* genome assembly. The top panel is informative of the position of
842 the gene (5'UTR as white rectangle, exon as black rectangle, 3'UTR as white arrow), T-DNA
843 and vector backbone (bb) (light and dark color arrows, respectively). Red arrows correspond
844 to the GABI-KAT T-DNA collection, blue arrows to the SAIL T-DNA collection, of which the
845 arrow points towards the right border, and green arrows to the JIC SM transposant library, of
846 which the arrow points towards the 3' end. Gray elements annotated on the T-DNA copies
847 and vector backbone sequences are I: mannopine synthase 1' 2' promoter, II: sulfonamide
848 resistance gene, III: region of the pUC18 vector, IV: ampicillin resistance gene, V: 35S
849 promoter, VI: BASTA resistance gene and VII: spectinomycin resistance gene, of which the
850 arrow points towards the 3' end. The second panel represents the individual reads aligned
851 onto the *de novo* genome assembly of the individual mutant lines, with methylated cytosines
852 (in the CG dinucleotide context) as black dots and non-methylated cytosines as blue dots.
853 The third panel shows the translation from genome coordinate space into a modified base
854 space consisting only of instances of the methylated motif (methylated cytosines in the CG
855 dinucleotide context). The fourth panel shows the raw log-likelihood ratio (LLR) plot of
856 cytosine methylation. The bottom panel shows a smoothed sliding window plot showing
857 methylation fraction across the region. Finally, shaded bands across the background of the
858 plot highlight the position of the T-DNA copies, dSpm element or vector backbone sequences
859 across the various panels.

860 **Figure 5.** Pairwise sequence alignments of the complete T-DNA (and dSpm element)
861 sequences of the most commonly used T-DNA consortia (and transposant) collections.
862 Sequence homology in the sense orientation is visible as a line from the upper left corner to
863 the bottom right corner of the dotplot. Sequence homology in the antisense orientation

864 (reverse complement) is visible as a line from the bottom left corner to the upper right corner
865 of the dotplot.

866 **Figure 6.** Proposed molecular mechanism for the initiation of *trans* T-DNA suppression.
867 Example of *trans* T-DNA suppression of the *ccr1-6* allele after a genetic cross of the *ugt72e3*
868 and *ccr1-6* T-DNA mutants sharing stretches of identical sequences. Initiation of *trans* T-DNA
869 suppression is mediated by the canonical RNA-directed DNA methylation (RdDM) pathway
870 (Matzke and Mosher, 2014; Wambui Mbichi et al., 2020). In short, double-stranded RNA
871 (dsRNA) is processed by dicer-like 3 (DCL3) into 24-nucleotide (nt) small interfering RNAs
872 (siRNAs) that are incorporated into argonaute 4 (AGO4) and base pair with the transcript
873 produced by RNA polymerase V (Pol V). In turn, AGO4 recruits domains rearranged
874 methyltransferase 2 (DRM2), which catalyzes the *de novo* CG methylation (Me) of
875 complementary DNA. It is hypothesized that the RNA duplex originates either from (A) a Pol
876 IV-produced transcript (e.g. *ugt72e3*) that is processed into dsRNA by RNA-dependent RNA
877 polymerase 2 (RDR2), which is part of the canonical RdDM pathway or (B and C) from RNA-
878 RNA annealing of transcript(s) made via Pol II. Primary transcripts generated by Pol II may
879 still contain T-DNA sequence(s) if not prematurely terminated. A primary transcript (e.g.
880 *ugt72e3*) containing a reverse complement T-DNA-derived sequence might interact (B) in *cis*
881 with a direct T-DNA-derived sequence (e.g. *ugt72e3*) to form a hairpin structure (Osabe et
882 al., 2017) or (C) in *trans* with a direct T-DNA-derived sequence from another locus (e.g. *ccr1-*
883 *6*) to form dsRNA (Jia et al., 2015). Note that the *ugt72e3* locus has a big deletion, resulting
884 in an intergenic region flanking the T-DNA insertion site. Also, the identical regions in the
885 GABI-Kat and SAIL T-DNA are in reverse orientation, affecting how transcripts from the
886 respective *ccr1-6* and *ugt72e3* loci can form dsRNA. In the gene models, black triangles,
887 black boxes, white boxes, solid lines and dotted lines represent the T-DNAs, exons, UTRs,
888 introns and intergenic regions respectively. Polymerases are in blue: Pol IV transcripts (30-
889 40 nt long: Zhai et al., 2015) and Pol V transcripts (approximately 200 nt in length:
890 Böhmdorfer et al., 2016), are much shorter than Pol II transcripts and are represented
891 accordingly. Other proteins involved in the initiation of *trans* T-DNA suppression are in red.
892 LB: T-DNA left border, RB: T-DNA right border and >/<: orientation from LB to RB.

893

894 **Table 1.** Crude cell wall residue (CWR) and lignin content of *ugt72e1,-e2,-e3 epiccr1-6*
 895 quadruple and *epiccr1-6* mutants. Measurements were performed on inflorescence stems of
 896 fully senesced plants. The CWR is expressed as a percentage of dry weight. Lignin content
 897 was determined with the acetyl bromide (AcBr) assay and expressed as a percentage of
 898 CWR. The data represent the mean values \pm standard deviation, n = 9. Different letters
 899 represent significant differences at the 0.05 significance level (ANOVA, Tukey *post-hoc*).

Line	CWR (% dry weight)	AcBr lignin (% CWR)
wild type	79.0 \pm 1.7 a	14.3 \pm 0.1 a
<i>ugt72e1,-e2,-e3</i>	78.0 \pm 2.4 a	13.8 \pm 0.2 a
<i>ccr1-6</i>	71.3 \pm 3.0 b	9.0 \pm 0.1 b
<i>ugt72e1,-e2,-e3 epiccr1-6</i>	74.1 \pm 4.6 b,c	10.7 \pm 0.1 c
<i>epiccr1-6 F4</i>	76.9 \pm 2.9 a,c	10.4 \pm 0.1 c
<i>epiccr1-6 F5</i>	77.0 \pm 2.0 a,c	10.8 \pm 0.2 c

900

901 **Table 2.** FA-derived lignin units in *ccr1*-CRISPR and *ugt72e1,-e2,-e3 ccr1*-CRISPR mutant
 902 lines. Lignin composition was determined with thioacidolysis. The data represent the mean
 903 values \pm standard deviation, n = 8. Different letters represent significant differences at the
 904 0.05 significance level (ANOVA, Tukey *post-hoc*). β -O-4-FA-I: G-CH=CH-COOH, β -O-4-FA-
 905 II: G-CHR-CH₂-COOH, bis- β -O-4-FA: G-CHR-CHR₂, where 'R' is a thioethyl group.

Line	β -O-4-FA-I (nmol g ⁻¹ AcBr lignin)	β -O-4-FA-II (nmol g ⁻¹ AcBr lignin)	bis- β -O-4-FA (nmol g ⁻¹ AcBr lignin)
wild type	6.31 \pm 0.75 a	3.47 \pm 0.97 a	31.66 \pm 13.12 a
<i>ugt72e1,-e2,-e3</i>	7.15 \pm 1.14 a	3.55 \pm 0.71 a	26.95 \pm 14.37 a
<i>ccr1</i> -CRISPR line 1	43.25 \pm 7.48 b	23.18 \pm 3.61 b	244.04 \pm 81.59 b
<i>ccr1</i> -CRISPR line 2	48.62 \pm 9.24 b	22.61 \pm 8.11 b	235.59 \pm 121.13 b
<i>ugt72e1,-e2,-e3 ccr1</i> -CRISPR line 1	39.59 \pm 10.73 b	12.64 \pm 6.50 c	132.60 \pm 76.66 b
<i>ugt72e1,-e2,-e3 ccr1</i> -CRISPR line 2	40.54 \pm 8.76 b	13.53 \pm 5.98 b,c	170.84 \pm 84.25 b

906