

1 **Regulation of poplar isoprenoid biosynthesis by methylerythritol**
2 **phosphate and mevalonic acid pathways interactions**

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4 Ali Movahedi¹, Hui Wei^{1,†}, Boas Pucker^{2,†}, Weibo Sun¹, Dawei Li¹, Liming Yang^{1,*}, and Qiang
5 Zhuge^{1,*}

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7 ¹ Co-Innovation Center for Sustainable Forestry in Southern China, Key Laboratory of Forest
8 Genetics & Biotechnology, Ministry of Education, College of Biology and the Environment,
9 Nanjing Forestry University, Nanjing 210037, China; ali_movahedi@njfu.edu.cn (A.M.);
10 15850682752@163.com (H.W.); czswb@njfu.edu.cn (W.S.); dwli@njfu.edu.cn (D.L.);
11 yangliming@njfu.edu.cn (L.Y.); qzhuge@njfu.edu.cn (Q.Z.)

12 ² Institute of Plant Biology, TU Braunschweig, Braunschweig, Germany; b.pucker@tu-
13 braunschweig.de (B.P.)

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16 * Correspondence: qzhuge@njfu.edu.cn; yangliming@njfu.edu.cn; Fax: +86-25-8542-8701

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18 † These authors contributed equally.

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32 **Summary**

33 Plants use two distinct isoprenoid biosynthesis routes: methylerythritol phosphate (MEP) and
34 mevalonic acid (MVA) pathways. The rate-limiting enzymes of the MEP pathway are 1-deoxy-
35 D-xylulose5-phosphate synthase (DXS) and 1-deoxy-D-xylulose5-phosphate reductoisomerase
36 (DXR). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the rate-limiting step in
37 the MVA pathway. Previously, overexpression of *Populus trichocarpa* *PtDXR* was found to
38 upregulate resistance against salt and drought stresses. We showed
39 while *PtHMGR* overexpressors (OEs) exhibited different MEP- and MVA-related gene
40 expressions than non-transgenic poplars (NT), the *PtDXR*-OEs revealed upregulated MEP-
41 related and downregulated MVA-related gene expressions. *PtDXR* and *PtHMGR*
42 overexpressions caused changes in MVA-derived trans-zeatin-riboside, isopentenyl adenosine,
43 castasterone, and 6-deoxocastasterone well as MEP-derived carotenoids and gibberellins.
44 In *PtHMGR*-OEs, the accumulated geranyl diphosphate synthase (*GPS*) and geranyl
45 pyrophosphate synthase (*GPPS*) transcript levels in the MEP pathway led to an accumulation
46 of MEP-derived isoprenoids. In contrast, upregulation of farnesyl diphosphate synthase (*FPS*)
47 expression in the MVA pathway contributed to increased levels of MVA-derived isoprenoids.
48 In addition, *PtHMGR*-OEs increased MEP-related *GPS* and *GPPS* transcript levels, expanded
49 MEP-derived isoprenoid levels, changed *FPS* transcript levels, and affected MVA-derived
50 isoprenoid yields. These results suggest that interaction exists between the MVA- and MEP-
51 pathways.

52 **Keywords:** MEP; MVA; poplar; terpenoids; Pathway interaction

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63 1. Introduction

64 Plants terpenoids include gibberellins (GAs), carotene, Lycopene, cytokinins (CKs),
65 strigolactones (GRs), and brassinosteroids (BRs) are produced through methylerythritol
66 phosphate (MEP) and mevalonic acid (MVA) pathways (Henry et al., 2015; van Schie et al.,
67 2006; Xie et al., 2008). The mentioned pathways are involved in plant growth, development,
68 and response to environmental changes (Bouvier et al., 2005; Kirby and Keasling, 2009). The
69 isopentenyl diphosphate isomerase (IDI) catalyzes the conversion of the isopentenyl
70 diphosphate (IPP) into dimethylallyl diphosphate (DMAPP), leading to provide the basic
71 materials for all isoprenoid productions (Hemmerlin, 2012; Lu et al., 2012; Zhang et al., 2019).
72 The produced IPP and DMAPP play essential roles in MEP and MVA pathways interactions
73 (Huchelmann et al., 2014; Liao et al., 2016). The MVA pathway reactions appear in the
74 cytoplasm, endoplasmic reticulum (ER), and peroxisomes (Cowan et al., 1997; Roberts, 2007),
75 producing sesquiterpenoids and sterols. The 3-hydroxy-3-methylglutaryl-CoA reductase
76 (HMGR), a rate-limiting enzyme in the MVA pathway, catalyzes 3-hydroxy-3-methylglutary-
77 CoA (HMG-CoA) to form MVA (Cowan et al., 1997; Roberts, 2007).

78 Reactions of the MEP pathway occur in the chloroplast and produce carotenoids, GAs,
79 and diterpenoids. 1-deoxy-D-xylulose5-phosphate synthase (DXS) and 1-deoxy-D-xylulose5-
80 phosphate reductoisomerase (DXR) are rate-limiting enzymes in the MEP pathway that
81 catalyze the conversion of D-glyceraldehyde3-phosphate (D-3-P) and pyruvate into 2-C-
82 methyl-D-erythritol4-phosphate (MEP) (Cordoba et al., 2009; Perreca et al., 2020; Wang et al.,
83 2012; Yamaguchi, 2018). Terpenoids like phytoalexin and volatile oils play essential roles in
84 plant growth, development, and disease resistance (Hain et al., 1993; Ren et al., 2008).
85 Photosynthetic pigments convert organic carbon into plant biomass (Esteban et al., 2015). In
86 addition to an extensive range of natural functions in plants, terpenoids also consider the
87 potential for biomedical applications. Paclitaxel is one of the most effective chemotherapy
88 agents for cancer treatment, and artemisinin is an anti-malarial drug (Kim et al., 2016a; Kong
89 and Tan, 2015).

90 Previous metabolic engineering studies have proposed strategies to improve the
91 production of specific metabolites in plants (Ghirardo et al., 2014; Opitz et al., 2014). For
92 example, PMT and H6H encoding the putrescine N-methyltransferase and hyoscyamine 6 β -

93 hydroxylase respectively produced significantly higher scopolamine in transgenic henbane
94 hairy root. Also, HCHL encoding p-hydroxycinnamoyl-CoA hydratase/lyase accumulated the
95 glucose ester of p-hydroxybenzoic acid (pHBA) in *Beta vulgaris* hairy root (Rahman et al., 2009;
96 Zhang et al., 2004). The 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) is the
97 second enzyme in the MVA pathway. Liao et al. (2018) confirmed that *HMGS* overexpression
98 of *Brassica juncea* upregulates carotenoid and phytosterol in tomatoes. HMGR has been
99 considered a critical factor in metabolically engineering terpenoids (Aharoni et al., 2005;
100 Dueber et al., 2009). In addition, *PgHMGR1* overexpression of ginseng increases ginsenosides
101 content, which is a necessary pharmaceutically active component (Kim, 2014).

102 Transgenic tobacco overexpressing the *Hevea brasiliensis* *HMGR* enhanced the
103 phytosterol levels (Schaller et al., 1995). It has been shown (Dai et al., 2011)
104 that *SmHMGR2* in *Salvia miltiorrhiza*, resulting in the improvement of squalene and
105 tanshinone contents. Moreover, *Arabidopsis thaliana* *HMGR1* (*AtHMGR1*) enhanced the
106 phytosterol levels in the first generation of transgenic tomatoes (Enfissi et al., 2005). While
107 the deaccumulation of *DXR* transcripts resulted in lower pigmentation and chloroplast
108 appearance defects, the upregulated *DXR* expression caused the MEP-derived plastid
109 isoprenoids to accumulate. Therefore, *DXR* can be genetically engineered to regulate the
110 content of terpenoids and expressed *DXR* in *Arabidopsis* and observed enhanced flux through
111 the MEP pathway (Carretero-Paulet et al., 2006). While the *A. thaliana* *DXR* overexpression
112 caused the diterpene anthiolimine to accumulate in *Salvia sclarea* hairy roots (Vaccaro et al.,
113 2014), the peppermint *DXR* overexpression resulted in essential oil inflation (about 50%) with
114 no significant variations in monoterpene composition (Mahmoud and Croteau, 2001).
115 Furthermore, previous studies have shown the exchange of metabolic intermediates included
116 in the MVA- and MEP-pathways through plastid membranes (Laule, 2003; Liao, 2006). In
117 summary, the overexpression of genes involved in the MEP- and MVA-pathways can change
118 the abundances or activities of related enzymes and metabolic products, causing a new
119 opportunity for plant breeding to enhance the accumulation of related metabolic products.

120 Poplars as an economic and energy species are widely used in industrial and agricultural
121 production. Its fast growth characteristics and advanced resources in artificial afforestation
122 play a vital role in the global ecosystem (Devappa, 2015).

123 This study investigates the poplar isoprenoid biosynthesis. We showed that *PtHMGR*-

124 *OEs* upregulated MVA- and MEP-related genes in the transcript levels. *PtDXR-OEs* have also
125 been shown involved in MVA-related genes down-regulation and MEP-related genes up-
126 regulation, resulting in increased terpenoid collection. These results indicate that the MEP is
127 a dominant pathway interacting with the MVA pathway and *HMGR* and *DXR* genes play key
128 regulation points in these pathways.

129 **2. Results**

130 2.1. *Isolation of the PtHMGR and PtDXR genes and characterization of transgenic* 131 *poplars*

132 The amino acid sequence of *PtHMGR* (Potri.004G208500.1) contains domains of other
133 HMGRs, including HMG-CoA-binding motifs (EMPVGYVQIP' and 'TTEGCLVA) and NADPH-
134 binding motifs (DAMGMNMV' and 'VGTVGGGT) (Ma et al., 2012) (Supplemental Figure 1).
135 Consequently, a phylogenetic tree with previously characterized HMGRs supported the
136 *PtHMGR* candidate identification (Supplemental Figure 2). The open reading frame of
137 the *PtHMGR* was amplified from *Populus trichocarpa* cDNA to clone in pEASY-T3 (TransGen
138 Biotech, China) and sequencing. The putative transgenic lines showed amplicons in PCR
139 identification compared to NT poplar (Supplemental Figure 3a). They also exhibited
140 increased *PtHMGR-OEs* expressions than NT (Supplemental Figure 3b), indicating successful
141 overexpression of *PtHMGR* in poplar. In addition, the *PtDXR* gene, which has been isolated,
142 sequenced, and analyzed previously by the authors (Xu et al., 2019), was used as the *PtDXR*-
143 *OEs* in this study.

144 2.2. *Effects of PtHMGR and PtDXR overexpressions on MVA- and MEP-related gene* 145 *expressions*

146 MVA-related genes *AACT*, *MVK*, and *MVD* except *HMGS* were significantly upregulated
147 in *PtHMGR-OE* transgenics than NT poplars (Figure 1a). While the expression of MEP-related
148 genes *DXS*, *DXR*, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (*HDS*), 1-hydroxy-
149 2-methyl-2-(E)-butenyl-4-diphosphate reductase (*HDR*), *IDI*, and *GPPS* were significantly
150 promoted in all *PtHMGR-OEs* transgenic poplars in comparing with NT,
151 the *GPS* overexpression was enhanced only by *PtHMGR-OE3* (Figure 1b). In addition, 2-C-
152 methyl-d-erythritol4-phosphate cytidyltransferase (*MCT*) and 4-diphosphocytidyl-2-C-
153 methyl-D-erythritol kinase (*CMK*) have been downregulated by *PtHMGR-OEs* (Figure 1b).

154 Moreover, while only *FPS* revealed significant upregulation by *PtDXR-OEs* in transgenics
155 comparing with NT, the other MVA-related genes *AACT*, *HMGS*, *HMGR*, and *MVK* were
156 considerably downregulated (Figure 1c). Finally, all MEP-related genes revealed significant
157 upregulation in *PtDXR-OEs* transgenic poplars (Figure 1d). *GPPS* and *HDS* genes exposed more
158 expressions induced by *DXR-OEs* than the other MEP-related genes (Figure 1d).

159 2.3. *PtHMGR overexpression influences the production of MVA and MEP derivatives*

160 β -carotene is a carotenoid synthesis that has been broadly used in the industrial
161 composition of pharmaceuticals and as food colorants, animal supplies additives, and
162 nutraceuticals. MVA-and MEP-pathways have been proved that are effective in the
163 biosynthesis of β -carotene (Yang, 2014). In addition, Lycopene is a carotenoid referring to C40
164 terpenoids and is broadly found in various plants, particularly vegetables and fruits. It has
165 been shown that MVA and MEP-pathways directly influence the biosynthesis production of
166 Lycopene (Kim et al., 2019; Wei et al., 2018). While Wille et al. (2004) showed that β -carotene
167 and Lutein are synthesized using intermediates from the MEP pathway, Opitz et al. (2014)
168 revealed that both MVA and MPE pathways contribute to producing isoprenoids such as β -
169 carotene and Lutein. HPLC-MS/MS has analyzed the quantity of MVA and MEP derivatives.
170 Our analyses revealed that *HMGR-OEs* caused a significant enhancement in Lycopene (an
171 average of ~ 0.08 ug/g), β -carotene (an average of ~ 0.33 ug/g), and Lutein (an average of ~
172 272 ug/g) production compared with NT poplars (~0.02, ~0.08, and ~100 ug/g respectively)
173 (Figure 2a, b, and c; Supplementary Figure 4). The ABA-related gene expressions also have
174 been calculated. Results revealed significant increased of *ZEP1*, 2, and 3 relative gene
175 expressions with the averages of ~2.85, ~4.67, and ~2.92 compared to NT with an average of
176 ~1 (Figure 2d). These results also shown meaningful enhancements of *NCED1*, 2, and 3
177 relative gene expressions with the averages of ~4.16, ~3.79, and ~3.4 compared to NT with an
178 average of ~1 (Figure 2e).

179 2.4. *Enhanced carotenoid levels in PtDXR-OE poplars*

180 The levels of the MEP-derived substances lycopene, β -carotene, and Lutein were
181 significantly increased in *PtDXR-OEs* with the averages of ~0.08, 0.22, 209.32 ug/g,
182 respectively compared to NT poplars (Figure 3a, b, and c; Supplemental figure 5). The analyses

183 of ABA-related gene expressions revealed significantly increased *ZEP1*, 2, and 3 relative gene
184 expressions with the averages of ~ 2.63 , ~ 2.38 , and ~ 3.86 compared to NT with an average of
185 ~ 1 (Figure 3d). These results also showed meaningful enhancements of *NCED2* and 3 relative
186 gene expressions with averages of ~ 2.25 and ~ 2.21 compared to NT with an average of ~ 1
187 (Figure 2e). These results revealed a decreased average in *NCED1* relative gene expression
188 with an average of ~ 0.66 compared to NT poplars.

189 2.5. *Other MVA and MEP derivatives*

190 The other MVA and MEP derivatives such as GAs, trans-zeatin-riboside (tZR), isopentenyl
191 adenosine (IPA), 6-deoxyocastasterone (DCS), and castasterone (CS) productions affected
192 by *PtHMGR*- and *PtDXR*-OEs have been analyzed. While Gibberellic acid (GA3) (a downstream
193 product of MEP) (an average of ~ 0.22 ng/g), tZR (an average of ~ 0.06 ng/g), IPA (an average
194 of ~ 0.59 ng/g), DCS (an average of 4.95 ng/g) revealed significantly more productions induced
195 by *HMGR*-OEs, the CS production (~ 0.095 ng/g) was decreased considerably compared to NT
196 poplars (~ 0.10 , ~ 0.03 , ~ 0.37 , ~ 1.50 , and ~ 0.20 ng/g respectively) (Figure 4a-j). These results
197 demonstrate that the *HMGR* gene interacts with MVA and MEP derivatives productions in
198 plants. On the other hand, the *PtDXR* overexpression significantly affected the contents of
199 MEP- and MVA-derived products except for CS. *PtDXR*-OEs showed a significant increase
200 ~ 0.276 ng/g in the GA3 content (Figure 4a and f). The tZR content represented a 10-fold
201 increase (~ 0.304 ng/g) affected by *PtDXR*-OEs compared to NT poplars (0.032 ng/g) (Figure 4b
202 and g). The content of IPA in *PtDXR*-OEs meaningfully increased ~ 0.928 ng/g, compared to
203 0.363 ng/g in NT poplars (Figure 4c and h) with a 3-fold increase. In addition, the DCS content
204 considerably increased to ~ 3.36 ng/g, comparing with ~ 1.50 ng/g in NT, representing a 3-fold
205 increase in *PtDXR*-OEs (Figure 4d and i). By contrast, the content of CS in *PtDXR*-OEs
206 significantly decreased (~ 0.137 ng/g) compared to NT poplar (0.203 ng/g), indicating
207 significant down-regulation in *PtDXR*-OEs (Figure 4e and j). The HPLC-MS/MS chromatograms
208 of GA, tZR, IPA, DCS, and CS standards are provided in Supplemental Figures 6–10.

209 2.6. *Phenotypic properties*

210 To figure out the effect of MVA-and MEP-pathway interactions and their changes
211 by *PtHMGR*-and *PtDXR*-OEs on plant growth and development, we decided to evaluate

212 phenotypic changes. Our results revealed a significant increase in GA3 contents in *PtDXR*-
213 *OEs* (Figure 4a) associated with a considerable rise in cytokinin tZR (Figure 4b), resulting in
214 significantly more development in stem length compared to *PtHMGR-OEs* and NT poplars
215 (Figure 5a and d). Regarding increasing ABA-related genes (*ZEP* and *NCED*) in *PtHMGR*-
216 *OEs* than *PtDXR-OEs* and NT poplars (Figure 5b) and also concerning insufficient increase
217 cytokinin tZR in *PtHMGR-OEs* comparing with NT poplars (Figure 4b), *PtHMGR* transgenics
218 showed a shorter stem length than *PtDXR* transgenics compared with NT poplars (Figure 5d).
219 We also figured out that only *PtDXR-OEs* revealed a few significant increases in stem diameters
220 than *PtHMGR-OEs* and NT poplars (Figure 5c and d).

221 3. Discussion

222 3.1. Characterization and evolutionary history of *HMGR*

223 Expression domains of *HMGR1* and *HMGR2* indicate a subfunctionalization. The
224 expression of *CaHMGR1* is temporary and tissue-specific, whereas that of *CaHMGR2* is
225 constitutive. *CaHMGR1* is only expressed in fruit tissues (pulp, endosperm, endocarp), flower
226 buds, and leaves during the initial developing steps. In contrast, *CaHMGR2* is expressed in all
227 tissues (flower buds, leaves, branches, and roots) and fruit tissues at various developing steps
228 (Tiski et al., 2011). *LcHMGR1* is most highly expressed during the early stages of fruit
229 development and regulates fruit size. The expression level of *LcHMGR1* is higher, and
230 expression lasts longer in larger fruits. *LcHMGR2* shows an expression peak during the late
231 stages of fruit development and is related to the biosynthesis of isoprenoid substances
232 required for cell elongation during that time (Rui et al., 2012). Previous studies have shown
233 that *NtHMGR2* is a stress-responsive gene (Hemmerlin et al., 2004; Merret et al., 2007). In
234 this study, high similarity of *PtHMGR* with other known *HMGR1* was observed.
235 Because *HMGR* is a conserved gene with a vital function in the MVA pathway, it can be used
236 as a reference gene to determine relationships among species.

237 3.2. *HMGR* overexpression results in upregulation of isoprenoid biosynthesis gene 238 expression

239 Liao et al. (2018) showed that overexpression of *BjHMGS1* affects the expression levels
240 of MEP- and MVA-related genes and slightly increases the transcript levels of *DXS* and *DXR* in

241 transgenic plants. However, *DXS*, *DXR*, *HDS*, and *HDR* expression levels have been
242 improved significantly in *PtHMGR-OE* poplars, while *MCT* and *CMK* are downregulated.

243 Similar to Liao et al. (2018) which the *BjHMGS1* overexpression in tomatoes significantly
244 increased the *GPS* and *GPPS* expressions, we exhibited that the *PtHMGR* overexpression
245 enhanced the farnesyl diphosphate synthase (*FPS*), *GPS*, and *GPPS* expressions may
246 stimulate the interaction between IPP and DMAPP, increasing the biosynthesis of plastidial
247 C15 and C20 isoprenoid precursors. Xu et al. (2012) showed that *HMGR* overexpression
248 in *Ganoderma lucidum* caused upregulated *FPS*, squalene synthase (*SQS*), or lanosterol
249 synthase (*LS*) mRNA expressions and developed the contents of ganoderic acid and
250 intermediates, including squalene and lanosterol. In addition, the *BjHMGS1* overexpression in
251 tomatoes significantly increased transcript levels of *FPS*, *SQS*, squalene epoxidase (*SQE*), and
252 cycloartenol synthase (*CAS*) (Liao et al., 2018). This study exhibited that except
253 for *HMGS* downregulating, the *AACT*, *MVK*, and *MVD* transcript levels were significantly
254 upregulated in *PtHMGR-OE* poplars. We revealed that these enhanced gene expressions
255 mainly were associated with the MVA-related genes contributing to the biosynthesis of
256 sesquiterpenes and other C15 and universal C20 isoprenoid precursors.

257 3.3. Overexpression of *PtDXR* affects MEP- and MVA-related genes

258 Zhang et al. (2018) showed that the *TwDXR* overexpression in *Tripterygium wilfordii*
259 increases the *TwHMGS*, *TwHMGR*, *TwFPS*, and *TwGPPS* expressions but decreases the *TwDXS*
260 expression. Moreover, Zhang et al. (2015) exhibited that the *NtDXR1* overexpression in
261 tobacco increases the transcript levels of eight MEP-related genes, indicating that the *NtDXR1*
262 overexpression led to upregulated MEP-related gene expressions. In *A. thaliana*, the *DXR*
263 transcript level changes do not affect *DXS* gene expression or enzyme accumulation, although
264 the *DXR* overexpression promotes MEP-derived isoprenoids such as carotenoids, chlorophylls,
265 and taxadiene (Carretero-Paulet et al., 2006).

266 On the other hand, the potato *DXS* overexpression in *A. thaliana* led to upregulation of
267 downstream *GGPPS* and phytoene synthase (*PSY*) genes (Henriquez et al., 2016). Furthermore,
268 (Simpson et al., 2016) exhibited that the *A. thaliana DXS* overexpression in *Daucus carota*
269 caused to enhance the *PSY* expression significantly.

270 In this study, while the *PtDXR-OEs* exposed higher MEP-related gene expressions than NT

271 poplars, the *PtDXR-OEs* revealed significant downregulated MVA-related gene expressions
272 than NT poplars. These findings illustrate that the MEP pathway regulates monoterpenes,
273 diterpenes, and tetraterpenoids biosynthesis and could affect the MVA pathway.

274 The diversity of biosynthetic pathways, the complexity of metabolic networks, and the
275 insufficient knowledge of gene regulation led to species-specific regulation patterns of MEP-
276 and MVA-related gene expression. One possible conclusion is that MEP- and MVA-related
277 genes often do not work alone but are co-expressed with upstream and downstream genes in
278 the MEP- and MVA- pathways to carry out a specific function.

279 3.4. *Overexpression of HMGR promotes the formation of GAs, and carotenoids in* 280 *plastids and accumulation of tZR, IPA, and DCS in the cytoplasm*

281 HMGR, as the rate-limiting enzyme in the MVA-pathway of plants, plays a critical role in
282 controlling the flow of carbon within this metabolic pathway. The upregulation
283 of *HMGR* significantly increases isoprenoid levels in plants. Overexpression of *HMGRs* of
284 different plant species has been reported to raise isoprenoids levels significantly. The
285 heterologous expression of *Hevea brasiliensis HMGR1* in tobacco increased the sterol content
286 and accumulated intermediate metabolites (Schaller et al., 1995). The *A. thaliana HMGR*
287 overexpression in *Lavandula latifolia* increased the levels of sterols in the MVA- and MEP-
288 derived monoterpenes and sesquiterpenes (Munoz-Bertomeu et al., 2007). In addition,
289 the *Salvia miltiorrhiza SmHMGR* overexpression in hairy roots developed MEP-derived
290 diterpene tanshinone (Kai et al., 2011). In our study, ABA synthesis-related genes
291 (*NCED1*, *NCED3*, *NCED6*, *ZEP1*, *ZEP2*, and *ZEP3*) and the contents of GA3 and carotenoids
292 were upregulated in *PtHMGR-OE* poplar seedlings. This finding suggests that
293 the *HMGR* overexpression may indirectly affect the biosynthesis of MEP-related isoprenoids,
294 including GA3 and carotenoids. The accumulation of MVA-derived isoprenoids including tZR,
295 IPA, and DCS was significantly elevated in *PtHMGR-OEs*, indicating
296 that *PtHMGR* overexpression directly influences the biosynthesis of MVA-related isoprenoids.
297 Therefore, the *HMGR* gene directly affects MVA-derived isoprenoids and indirectly affects the
298 content of MEP-derived isoprenoids by changing the expression levels of MEP-related genes.

299 3.5. *Higher levels of MEP- and MVA-derived products in PtDXR-OE seedlings*

300 DXR is the rate-limiting enzyme in the MEP pathway and an essential regulatory step in

301 the cytoplasmic metabolism of isoprenoid compounds (Takahashi et al., 1998). Mahmoud and
302 Croteau (2001) revealed that overexpression of *DXR* in *Mentha piperita* promoted the
303 synthesis of monoterpenes in the oil glands and increased the production of essential oil yield
304 by 50%. However, the up-regulation of *DXR* expression did not lead to change in the complex
305 oil composition significantly. Hasunuma et al. (2008) exhibited that overexpression of
306 *Synechocystis sp.* strain PCC6803 *DXR* in tobacco resulted in increased levels of β -carotene,
307 chlorophyll, antheraxanthin, and Lutein. Xing et al. (2010) showed that the *A. thaliana dxr*
308 mutants caused to lack of GAs, ABA, and photosynthetic pigments (REF57). These mutants
309 showed pale sepals and yellow inflorescences (Xing et al., 2010). In our study, the relatively
310 higher abundance of GA3 and carotenoids in *PtDXR-OE* poplar seedlings indicated an effect of
311 *DXR* overexpression. Combined with the result described above of increased *DXS*, *HDS*, *HDR*,
312 *MCT*, *CMK*, *FPS*, *GPS*, and *GPPS* expression levels, we postulate that overexpression of *DXR* not
313 only affects the expression levels of MEP-related genes but also changes the field of GA3, and
314 carotenoids.

315 3.6. *Interaction between the MVA and MEP pathways*

316 Although the substrates of MVA- and MEP-pathways differ, there are common
317 intermediates like IPP and DMAPP (Figure 6). Blocking only the MVA or the MEP pathway,
318 respectively, does not entirely prevent the biosynthesis of terpenes in the cytoplasm or
319 plastids, indicating that some MVA and MEP pathways products can be transported and/or
320 move between cell compartments (Aharoni et al., 2003; Aharoni et al., 2004; Gutensohn et
321 al., 2013). For example, it has been shown that the transferring IPP from the chloroplast to
322 cytoplasm observed through ¹³C labeling, indicating that plentiful IPP is available for use in
323 the MVA-pathway to produce terpenoids (Ma et al., 2017). In addition, segregation between
324 the MVA- and MEP-pathways is limited and might exchange some metabolites over the plastid
325 membrane (Laule, 2003). Kim et al. (2016b) used clustered, regularly interspaced short
326 palindromic repeats (CRISPR) technology to reconstruct the lycopene synthesis pathway and
327 control the flow of carbon in the MEP-and MVA-pathways. The results showed that the
328 expression of MVA-related genes was reduced by 81.6%, but the lycopene yield was
329 significantly increased. By analyzing gene expression levels and metabolic outcome
330 in *PtHMGR*-and *PtDXR-OEs*, we discovered that the correlation might exist between MVA- and

331 MEP-related genes with MVA- and MEP-derived products, which are not restricted to crosstalk
332 between IPP and DMAPP (Figure 6).

333 On the one hand, overexpression of *PtDXR* affected the transcript levels of MEP-related
334 genes and the contents of MEP-derived isoprenoids, including GAs and carotenoids. The
335 diminished accumulation of MVA-related gene products causes a reduction in the yields of
336 MVA-derived isoprenoids (including CS) but leads to increasing tZR, IPA, and DCS contents. We
337 hypothesize that IPP and DMAPP produced by the MEP pathway could enter the cytoplasm to
338 compensate for the lack of IPP and DMAPP, and the IPP and DMAPP as the precursors of the
339 MVA pathway are used to guide the synthesis of MVA-derived products. On the other
340 hand, *PtHMGR-OEs* exhibited higher transcript levels of *AACT*, *MVK*, and *MVD* and
341 higher *DXS*, *DXR*, *HDS*, and *HDR* than NT poplars, resulting in effect both MEP- and MVA-
342 related gene expressions. We successfully demonstrated that manipulation of *HMGR* in the
343 poplar MVA-pathway results in dramatically enhanced yields of GAs and carotenoids. This
344 result illustrates that cytosolic *HMGR* overexpression expanded plastidial GPP- and GGPP-
345 derived products, such as carotenoids. Therefore, this study provides hints that crosstalk
346 between the MVA- and MEP-pathways increased the expression levels
347 of *GPS* and *GPPS* in *PtHMGR-OEs*, and elevated the contents of GA3 and carotenoids.
348 Moreover, changes in MEP- and MVA-related gene expressions affect MVA- and MEP-derived
349 isoprenoids. Identification of the molecular mechanism holding this crosstalk requires further
350 investigation.

351 In conclusion, overexpression of *PtHMGR* in poplars caused the accumulation of MVA-
352 derived isoprenoids and MEP-derived substances. The advanced insights into the regulation
353 of MVA- and MEP-pathways in poplar add to the knowledge about these pathways in
354 *Arabidopsis*, tomato, and rice. In *PtHMGR-OE* poplars, most MEP- and MVA-related genes
355 associated with the biosynthesis of isoprenoid precursors were upregulated. In *PtDXR*-
356 *OE* poplars, elevated contents of GAs, carotenoids, and GRs were attributed to increased
357 expression of MEP-related genes as well as plastidial *GPP* and *GGPP*. Together, these results
358 show that manipulating *PtDXR* and *PtHMGR* is a novel strategy to influence poplar isoprenoids.

359 3.7. *The impressed crosstalk between MVA- and MEP-pathways by PtHMGR-* 360 *and PtDXR-OEs influence plant growth and developments*

361 It has been shown that Abscisic acid (ABA) and GA3 perform essential functions in cell
362 division, shoot growth, and flower induction (Xing et al., 2016). It has also been demonstrated
363 that the cytokinin tZR, a variety of phytohormones, perform a crucial function as root to shoot
364 signals, directing numerous developmental and growth processes in shoots (Abul et al., 2010;
365 Sakakibara, 2006). Regarding these findings, we showed how the interactions between MVA-
366 and MEP-pathways and their changes affected by some stimulators (*HMGR*-and *DXR-OEs*)
367 influenced plant growth, especially in stem length. Finally, We figured out that the gibberellic
368 acid and cytokinin may be more effective in plant growth than inhibiting by ABA, causing
369 higher *PtDXR-OEs* than *PtHMGR-OEs* compared with NT poplars.

370 **4. Materials and Methods**

371 4.1. *Plant materials and growth conditions*

372 Non-transgenic *P. trichocarpa* and *Populus × euramericana* cv. 'Nanlin 895' plants were
373 cultured in half-strength Murashige and Skoog (1/2 MS) medium (pH 5.8) under conditions of
374 24°C and 74% humidity (Movahedi et al., 2015). Subsequently, NT and transgenic poplars were
375 cultured in 1/2 MS under long-day conditions (16 h light/8 h dark) at 24°C for 1 month
376 (Movahedi et al., 2018).

377 4.2. *PtHMGR and PtDXR genes isolation and vector construction*

378 To produce cDNA, total RNA was extracted from *P. trichocarpa* leaves and processed with
379 PrimeScript™ RT Master Mix, a kind of reverse transcriptase (TaKaRa, Japan). Forward and
380 reverse primers (Supplemental Table 1: *PtHMGR-F* and *PtHMGR-R*) were designed, and the
381 open reading frame (ORF) of *PtHMGR* was amplified via PCR. We then used the total volume
382 of 50µl including 2 µl primers, 2.0 µl cDNA, 5.0 µl 10 × PCR buffer (Mg²⁺), 4µl dNTPs (2.5 mM),
383 0.5 µl rTaq polymerase (TaKaRa, Japan) for the following PCR reactions: 95°C for 7 min, 35
384 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min. Subsequently,
385 the product of the *PtHMGR* gene was ligated into the pEASY-T3 vector (TransGen Biotech,
386 China) based on blue-white spot screening, and the *PtHMGR* gene was inserted into the vector
387 pGWB9 (Song et al., 2016) using Gateway technology (Invitrogen, USA). On the other hand, all

388 steps to generate cDNA, RNA extraction, PCR, pEASY-T3 ligation, and vector construction
389 (pGWB9-PtDXR) of *PtDXR* have been carried out according to Xu et al. (2019).

390 4.3. *Creation of phylogenetic tree*

391 We applied the ClustalX for multiple sequence alignment of HMGR proteins, and
392 MEGA5.0 software was used to construct a phylogenetic tree using 1000 bootstrap replicates.
393 The amino acid sequences of HMGR from *Populus trichocarpa*, *Arabidopsis thaliana*,
394 *Gossypium raimondii*, *Malus domestica*, *Manihot esculenta*, *Oryza sativa*, *Prunus persica*,
395 *Theobroma cacao*, and *Zea mays* were obtained from the National Center for Biotechnology
396 Information database (<https://www.ncbi.nlm.nih.gov/>) and Phytozome ([https://phytozome-
397 next.jgi.doe.gov/](https://phytozome-next.jgi.doe.gov/)).

398 4.4. *Transgenic poplars: generation and confirmation*

399 *Agrobacterium tumefaciens* var. EHA105 was used for the infection of poplar leaves and
400 petioles (Movahedi et al., 2014). Poplar buds were screened on differentiation MS medium
401 supplemented with 30 µg/mL Kanamycin (Kan). Resistant buds were planted in bud elongation
402 MS medium containing 20 µg/mL Kan and transplanted into 1/2 MS medium including 10
403 µg/mL Kan to generate resistant poplar trees. Genomic DNA has been extracted from putative
404 transformants one-month-old leaves grown on a kanamycin-containing medium using
405 TianGen kits (TianGen BioTech, China). The quality of the extracted genomic DNA (250–350
406 ng/µl) was determined by a BioDrop spectrophotometer (UK). PCR was carried out using
407 designed primers (Supplementary Table 1: CaMV35S as the forward and PthMGR as the
408 reverse), Easy Taq polymerase (TransGene Biotech), and 50 ng of extracted genomic DNA as a
409 template to amplify about 2000 bp. In addition, total RNA was extracted from these one-
410 month-old leaves to produce cDNA, as mentioned above. These cDNA then were applied to
411 reverse transcription-quantitative PCR (RT-qPCR) (Supplemental Table 1: PthMGR forward and
412 reverse) for comparing the transformants *PthMGR-OEs* expressions with NT poplars and
413 transforming confirmation.

414 4.5. *Phenotypic properties evaluation*

415 To evaluate phenotypic changes, we selected 45-day-old poplars from PthMGR-and

416 PtDXR-OEs and NT poplars. We then simultaneously calculated the stem lengths (mm) and
417 stem diameters (mm) every day and recorded them. All recorded were analyzed by GraphPad
418 Prism 9, applying ANOVA one way (Supplemental Table 2).

419 4.6. *Analyses via qRT-PCR*

420 12-month-old *PtDXR-OEs* (Xu et al., 2019) and *PtHMGR-OE* poplars (Soil-grown poplars)
421 have been used to extract total RNA. The qRT-PCR was performed to identify MVA- and MEP-
422 related gene expression levels in NT, *PtDXR-OE*, and *PtHMGR-OE* poplars. The qRT-PCR was
423 served with a StepOne Plus Real-time PCR System (Applied Biosystems, USA) and SYBR Green
424 Master Mix (Roche, Germany). Poplar *Actin* (*PtActin*) (XM-006370951.1) was previously tested
425 as a reference gene for this experiment (Zhang et al., 2013). The following conditions were
426 used for qRT-PCR reactions: pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at
427 95°C for 15 s, and a chain extension at 60°C for 1 min. Three independent experiments were
428 conducted using gene-specific primers (Supplemental Table 1: PtHMGR forward and reverse).

429 4.7. *Metabolite analyses via high-performance liquid chromatography-tandem* 430 *mass spectrometry*

431 The isopropanol/acetic acid extraction method extracted poplar endogenous hormones
432 from NT, *PtDXR-OE*, and *PtHMGR-OE* leaves. GAs and CKs were extracted from, and then HPLC-
433 MS/MS (Qtrap6500, Agilent, USA) was used to quantify levels of GAs, zeatin, tZR, and IPA. Also,
434 methanol considered as solvent was used to extract 5-Deoxystrigol (5-DS), CS, and DCS, and
435 HPLC-MS/MS (Agilent1290, AB; SCIEX-6500Qtrap, Agilent; USA) was also used to determine
436 the contents of 5-DS, CS, and DCS. In addition, acetone, as a solvent, was used to isolate the
437 carotenoid component of poplar leaves. To identify the carotenoid contents, the peak areas
438 of carotenoids analyzed by HPLC (Symmetry Shield RP18, Waters, USA) were used to draw
439 standard carotenoid curves, including β -carotene, Lycopene, and Lutein. Also, the HPLC was
440 used to determine the contents of carotenoids, including β -carotene, Lycopene, and Lutein in
441 NT and OE lines.

442 **Author contributions**

443 A.M. and H.W. conceived, planned, and coordinated the project, performed data analysis,
444 wrote the draft, and finalized the manuscript. B.P. validated and contributed to data analysis
445 and curation, revised and finalized the manuscript. W.S. and D.L. reviewed and edited the

446 manuscript. L.Y. and Q.Z. coordinated, contributed to data curation, finalized and funded this
447 research. A.M., H.W., and B.P. contributed equally as the first author.

448 **Conflict of interest**

449 The authors declare that they have no conflict of interest.

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- 646

647 **6. Figure legends**

648 **Figure 1 | MEP- and MVA-related genes analyses in overexpressed *PtHMGR*- and *PtDXR-O***

649 ***Es poplars*. a**, MVA-related genes *AACT*, *HMGS*, *MVK*, *MVD*, and *FPS* affected by *PtHMGR* over

650 expressing. **b**, MEP-related genes *DXS*, *MCT*, *CMK*, *HDS*, *HDR*, *IDI*, *GPS*, *GPPS*, and *DXR* affected

651 d by *PtHMGR* overexpressing. **c**, MVA-related genes *AACT*, *HMGS*, *HMGR*, *MVK*, *MVD*, and *FP*

652 *S* affected by *PtDXR* overexpressing. **d**, MEP-related genes *DXS*, *DXR*, *MCT*, *CMK*, *HDS*, *HDR*, *ID*

653 *I*, *GPS*, and *GPPS* affected by *PtDXR* overexpressing. *PtActin* was used as an internal reference

654 in all repeats; * P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001; Three independent repli

655 cations were performed in this experiment.

656 **Figure 2 | HPLC-MS/MS content analyses of lycopene, β -carotene, Lutein, and real-time PCR**

657 **of *ZEP* and *NCED* genes family.** HPLC-MS/MS content analyses have been performed to show

658 the effect of *PtHMGR-OEs* on **a**, lycopene **b**, β -carotene, and **c**, lutein. Relative expressions

659 have been analyzed affected by *PtHMGR-OEs* comparing with NT poplars of **d**, *ZEP*,

660 and **e**, *NCED* genes family. Bars represent mean \pm SD (n = 3); Stars reveal significant differences,

661 * P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001; Three independent experiments were

662 performed in these analyses.

663 **Figure 3 | HPLC-MS/MS content analyses of lycopene, β -carotene, Lutein, and real-time PCR**

664 **of *ZEP* and *NCED* genes family.** HPLC-MS/MS content analyses have been performed to show

665 the effect of *PtDXR-OEs* on **a**, lycopene **b**, β -carotene, and **c**, lutein. Relative expressions have
666 been analyzed affected by *PtDXR-OEs* comparing with NT poplars of **d**, *ZEP*,
667 and **e**, *NCED* genes family. Bars represent mean \pm SD (n = 3); Stars reveal significant differences,
668 * P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001; Three independent experiments were
669 performed in these analyses.

670 **Figure 4 | HPLC-MS/MS content analyses of MEP- and MVA-derived isoprenoids. a,b,c,d,**
671 **and e**, Violin plots reveal the contents of isoprenoids GA3, tZR, IPA, DCS, and CS obtained from
672 MEP- and MVA-pathways influenced by *PtHMGR-* and *PtDXR-OEs*. **f,g,h,i, and j**, the column
673 plots reveal the effect of *PtHMGR-OE3* and -7 and *PtDXR-OE1* and -3 on the mentioned above
674 isoprenoids separately; NT poplars have been used as the control. Bars represent mean \pm SD
675 (n = 3); Stars reveal significant differences, *P < 0.05, **P < 0.01, ***P < 0.001, ****P <
676 0.0001. k,l,m,n, and o, represent the HPLC-MS/MS chromatogram content analyses of GA3,
677 tZR, IPA, DCS, and CS, respectively affected by *PtHMGR-* and *PtDXR-OEs* comparing with NT
678 poplars.

679 **Figure 5 | Phenotypic changes resulted by affected MVA- and MEP- pathway interactions in**
680 **45-day-old poplars. a**, Mean comparisons of stem lengths revealed significantly higher
681 lengths *PtDXR-OEs* than NT poplars compared with *PtHMGR-OEs*. *PtHMGR* transgenics also
682 revealed significantly higher lengths than NT poplars. **b**, Mean comparisons of *ZEP* and *NCED*
683 relative expressions between *PtHMGR-and PtDXR-OEs* compared to NT poplars. **c**, Mean
684 comparisons of stem diameters revealed less significant differences between *PtDXR-OEs* and
685 NT poplars. Stars reveal significant differences, *P < 0.05, **P < 0.01, ***P < 0.001, ****P <
686 0.0001. **d(I)**, The *PtDXR* transgenic revealed a higher stem length than *PtHMGR-OEs* and NT
687 poplars. **d(II)**, The *PtHMGR* transgenic presents an insignificantly more stem development
688 than NT poplar. **d(III)**, NT poplar was used as a control; Scale bar represents 1 cm.

689 **Figure 6 | The interactions between MEP- and MVA-pathways.** The IPP and DMAPP are
690 considered the common precursors of the MEP- and MVA-pathways between cytoplasm and
691 plastid. In addition, the putative communication generates between MVA- and MEP-related
692 genes and MVA- and MEP-derived products. MVA: mevalonic acid, MEP: methylerythritol
693 phosphate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, AACT:
694 acetoacetyl CoA thiolase, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMG-CoA: 3-
695 hydroxy-3-methylglutaryl-CoA, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, MVK:

696 mevalonate kinase, MVD: mevalonate5-diphosphate decarboxylase, IPP: isopentenyl
697 diphosphate, IDI: IPP isomerase, GPP: geranyldiphosphate, FPP: farnesylidiphosphate, GPS:
698 geranyl phosphate synthase, FPS: farnesyl-diphosphate synthase, GPPS: geranyl diphosphate
699 synthase, GGPPS: geranyl geranyl diphosphate synthase, DXS: 1-deoxy-D-xylulose5-phosphate
700 synthase, DXP: 1-deoxy-D-xylulose5-phosphate, DXR: 1-deoxy-D-xylulose5-phosphate
701 reductoisomerase, HDS: 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphate synthase, HDR: 1-
702 hydroxy-2-methyl-2-(E)-butenyl4-diphosphate reductase, MCT: MEP cytidyltransferase,
703 CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase.

704 **Supplemental figures and table**

705 **Supplemental Figure 1 | Amino acid sequences alignment of PtHMGR protein and other**
706 **known HMGR proteins.** *A. thaliana* (NP_177775.2), *G. hirsutum* (XP_016691783.1), *M.*
707 *domestica* (XP_008348952.1), *M. esculenta* (XP_021608133.1), *P. persica* (XM_020569919.1),
708 *O. sativa* (XM_015768351.2), *T. cacao* (XM_007043046.2), *Z. mays* (PWZ28886.1). The HMG-
709 CoA and NADPH binding domains are indicated in red rectangular.

710 **Supplemental Figure 2 | Construction of a phylogenetic tree based on the HMGR sequences**
711 **of various species.** Accession numbers of the HMGR obtained from Phytozome are as follows:
712 *A. thaliana* (AT1G76490 and AT2G17370) , *P. trichocarpa* (Potri.011G145000,
713 Potri.005G257000, Potri.004G208500, Potri.001G457000, Potri.009G169900 and
714 Potri.002G004000), *Gossypium raimondii* (Gorai.008G013000, Gorai.002G146000,
715 Gorai.002G014700, Gorai.005G215800, Gorai.012G138100, Gorai.005G215500,
716 Gorai.005G215600 and Gorai.005G215700), *Malus domestica* (MDP0000157996,
717 MDP0000268909, MDP0000372490, MDP0000251253 and MDP0000312032), *Manihot*
718 *esculenta* (Manes.15G114100, Manes.01G157500, Manes.03G096600, Manes.02G116900
719 and Manes.05G128600), *Oryza sativa* (LOC_Os09g31970, LOC_Os08g40180 and
720 LOC_Os02g48330), *Prunus persica* (Prupe.7G187000, Prupe.7G187500 and Prupe.8G182300),
721 *Theobroma cacao* (Thecc1EG000025, Thecc1EG007601 and Thecc1EG034814), and *Zea mays*
722 (GRMZM2G393337, GRMZM2G058095, GRMZM2G136465, GRMZM2G001645 and
723 GRMZM2G043503).

724 **Supplemental Figure 3 | Molecular identification of PtHMGR-OEs.** (A) PCR identification of
725 *PtHMGR* in *PtHMGR-OEs* and NT poplars. Lane M: 15K molecular mass marker (TransGen,
726 China); lane 1: genome DNA from WT as negative control; lanes 2–9: genome DNA from

727 *PtHMGR-OE* lines **(B)** qRT-PCR identification of the transcript levels of *PtHMGR* in *PtHMGR-*
728 *OEs* and NT poplars. Three independent experiments were performed; Stars reveal significant
729 differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

730 **Supplemental Figure 4** | HPLC chromatograms of analyzing the contents of **(A)** β -carotene, **(B)**
731 lycopene, and **(C)** lutein in NT poplars and *PtHMGR-OEs*.

732 **Supplemental Figure 5** | HPLC-MS/MS chromatogram analyses of the contents of **(A)** GA3, **(B)**
733 TZR, **(C)** IPA, **(D)** DCS, and **(E)** CS affected by *PtHMGR-OE3* and -7 comparing with NT poplars.

734 **Supplemental Figure 6** | **Chromatogram analyses of GA3 standards via HPLC-MS/MS.** The
735 chromatogram of standard GA3 at **(A)** 0.1, **(B)** 0.2, **(C)** 0.5, **(D)** 2, **(E)** 5, **(F)** 20, **(G)** 50, and **(H)**
736 200 ng/mL concentrations. **(I)** Equations for the GA3 standard curves.

737 **Supplemental Figure 7** | **Chromatogram analyses of tZR standards via HPLC-MS/MS.** The
738 chromatogram of standard tZR at **(A)** 0.1, **(B)** 0.2, **(C)** 0.5, **(D)** 2, **(E)** 5, **(F)** 20, **(G)** 50, and **(H)**
739 200 ng/mL concentrations. **(I)** Equations for the tZR standard curves.

740 **Supplemental Figure 8** | **Chromatogram analyses of IPA standards via HPLC-MS/MS.** The
741 chromatogram of standard IPA at **(A)** 0.2, **(B)** 0.5, **(C)** 2, **(D)** 5, **(E)** 20, **(F)** 50, and **(G)** 200 ng/mL
742 concentrations. **(H)** Equations for the IPA standard curves.

743 **Supplemental Figure 9** | **Chromatogram analyses of DCS standards via HPLC-MS/MS.** The
744 chromatogram of standard DCS at **(A)** 0.5, **(B)** 2, **(C)** 10, **(D)** 20, and **(E)** 50 ng/mL concentrations.
745 **(F)** Equations for the DCS standard curves.

746 **Supplemental Figure 10** | **Chromatogram analyses of CS standards via HPLC-MS/MS.** The
747 chromatogram of standard CS at **(A)** 0.5, **(B)** 5, **(C)** 10, **(D)** 20, and **(E)** 50 ng/mL concentrations.
748 **(F)** Equations for the CS standard curves.

749 **Supplemental Table 1** | Primers were used in this study.

750 **Supplemental Table 2** | Table of data analyses used in phenotypic changes evaluation. **a**, Stem
751 diameter data analyses. **b**, Stem length data analyses.

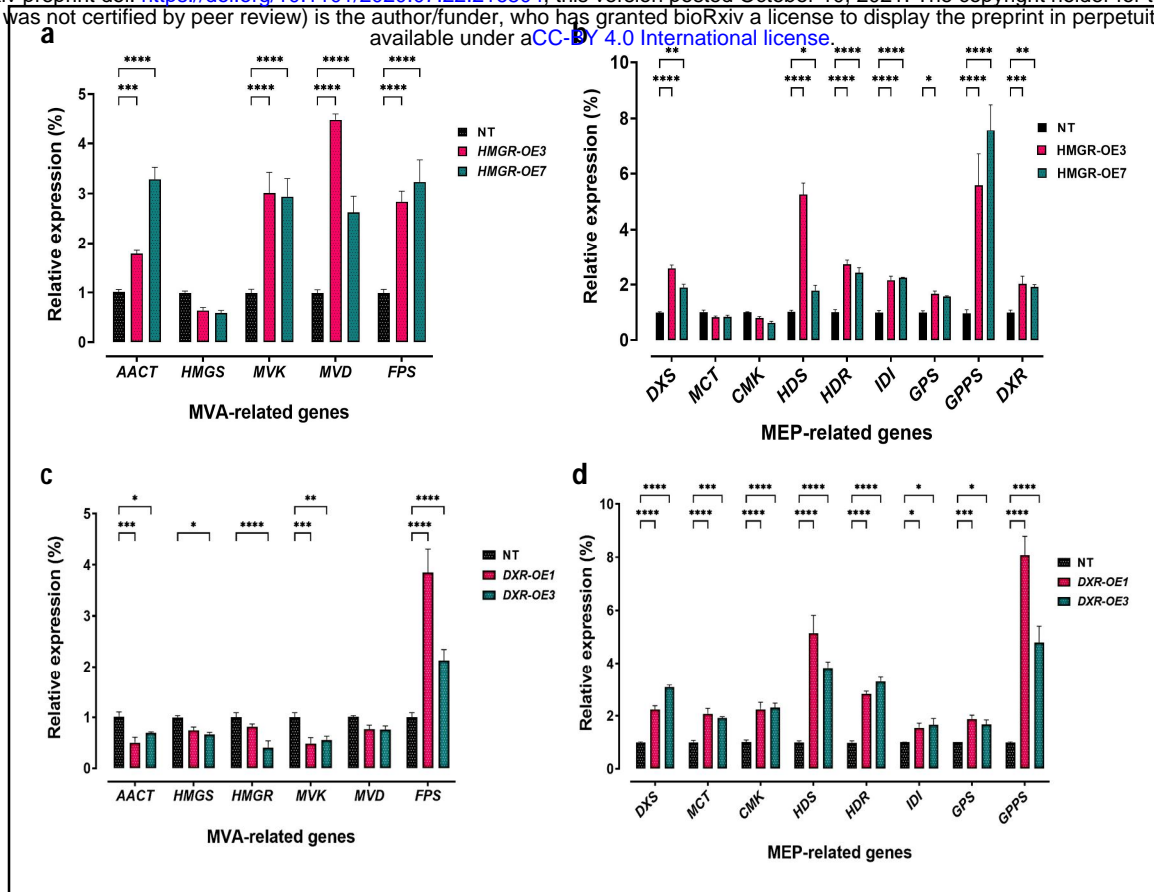


Figure 1 | MEP- and MVA-related genes analyses in overexpressed *PtHMGR*- and *PtDXR*-OEs poplars. a, MVA-related genes *AACT*, *HMGS*, *MVK*, *MVD*, and *FPS* affected by *PtHMGR* overexpressing. **b**, MEP-related genes *DXS*, *MCT*, *CMK*, *HDS*, *HDR*, *IDI*, *GPS*, *GPPS*, and *DXR* affected by *PtHMGR* overexpressing. **c**, MVA-related genes *AACT*, *HMGS*, *HMGR*, *MVK*, *MVD*, and *FPS* affected by *PtDXR* overexpressing. **d**, MEP-related genes *DXS*, *DXR*, *MCT*, *CMK*, *HDS*, *HDR*, *IDI*, *GPS*, and *GPPS* affected by *PtDXR* overexpressing. *PtActin* was used as an internal reference in all repeats; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; Three independent replications were performed in this experiment.

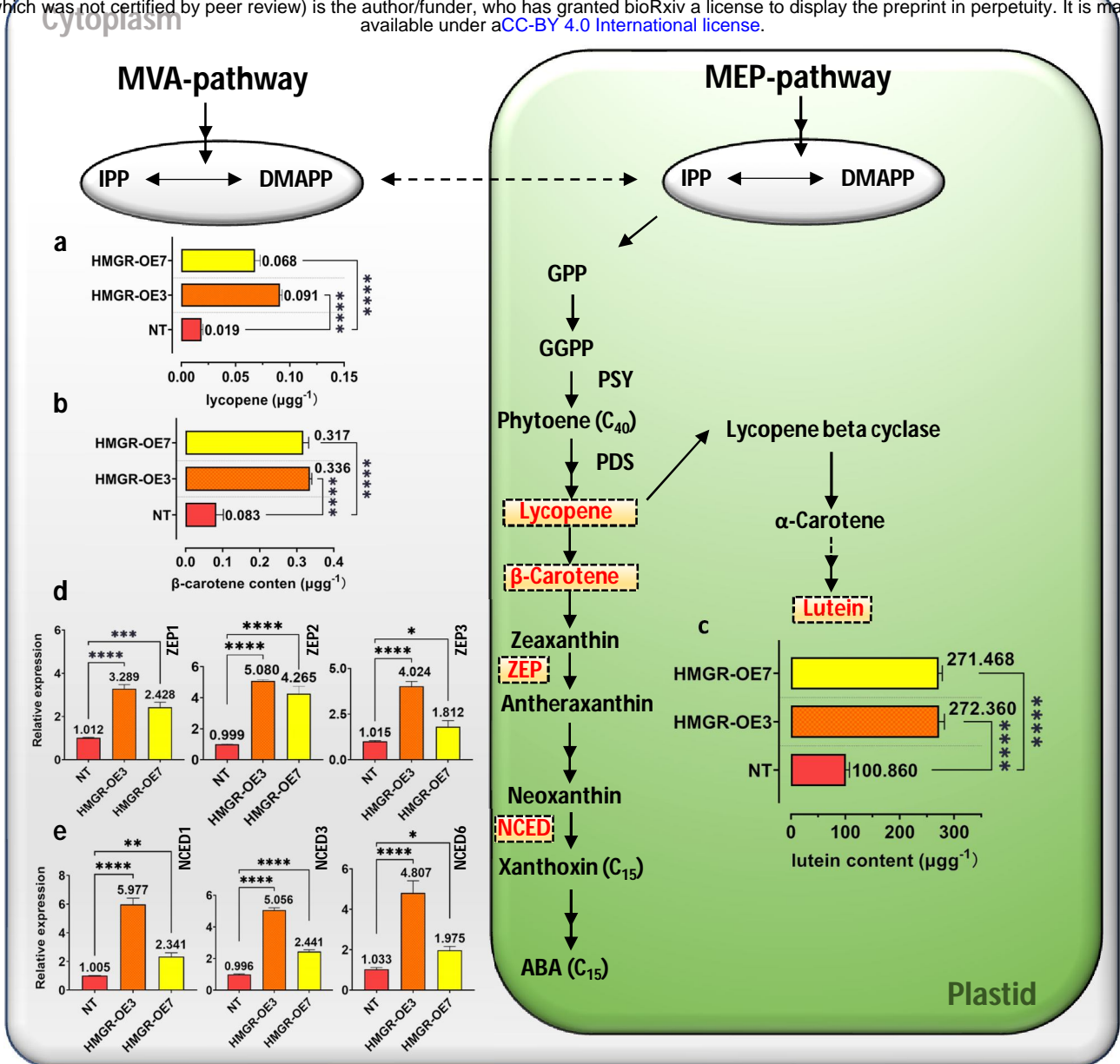


Figure 2 | HPLC-MS/MS content analyses of lycopene, β -carotene, lutein, and real-time PCR of ZEP and NCED genes family. HPLC-MS/MS content analyses have been performed to show the effect of *PtHMGR-OEs* on **a**, lycopene **b**, β -carotene, and **c**, lutein. Relative expressions have been analyzed affected by *PtHMGR-OEs* comparing with NT poplars of **d**, ZEP, and **e**, NCED genes family. Bars represent mean \pm SD (n = 3); Stars reveal significant differences, * P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001; Three independent experiments were performed in these analyses.

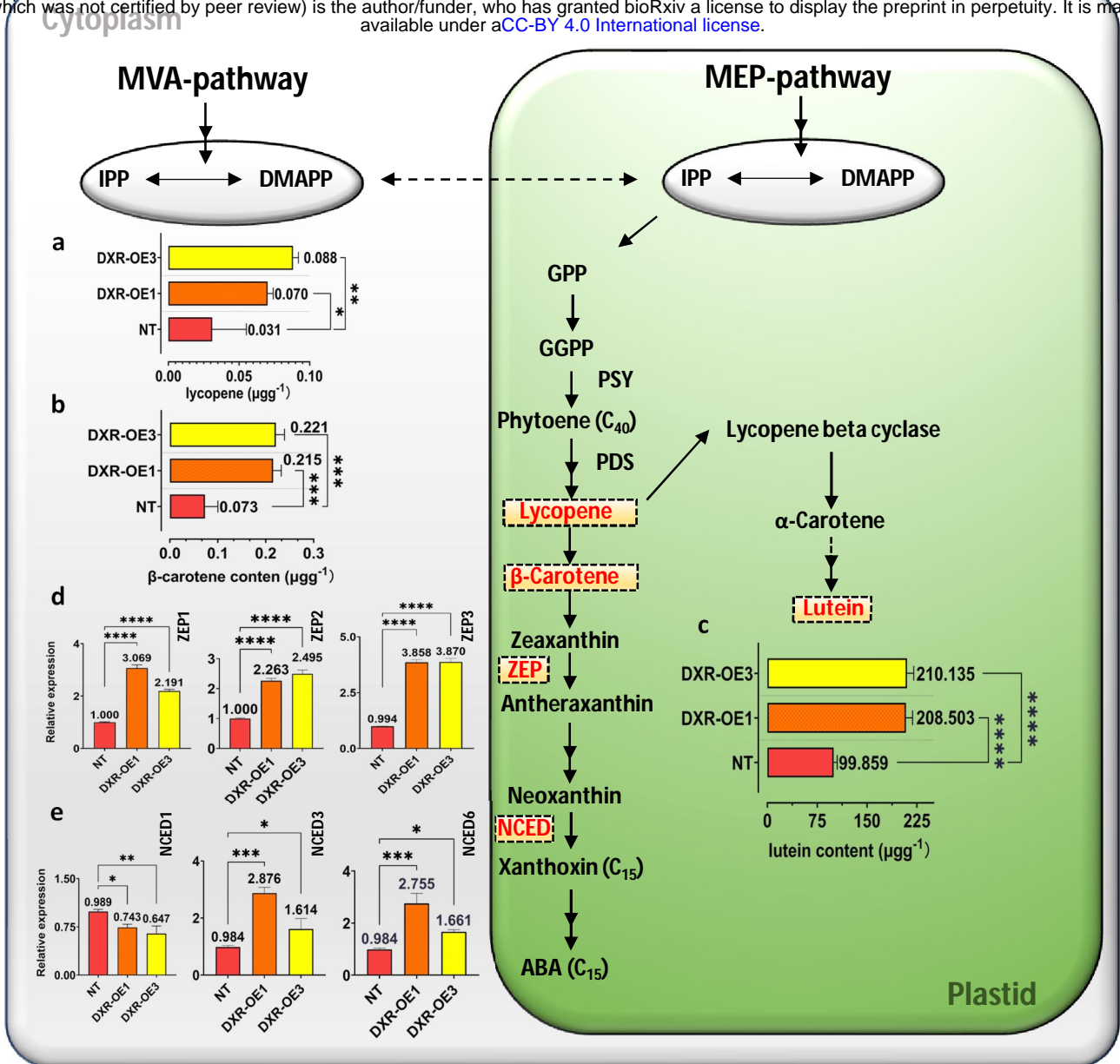


Figure 3 | HPLC-MS/MS content analyses of lycopene, β -carotene, lutein, and real-time PCR of ZEP and NCED genes family. HPLC-MS/MS content analyses have been performed to show the effect of *PtDXR*-OEs on **a**, lycopene **b**, β -carotene, and **c**, lutein. Relative expressions have been analyzed affected by *PtDXR*-OEs comparing with NT poplars of **d**, ZEP, and **e**, NCED genes family. Bars represent mean \pm SD (n = 3); Stars reveal significant differences, * P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001; Three independent experiments were performed in these analyses.

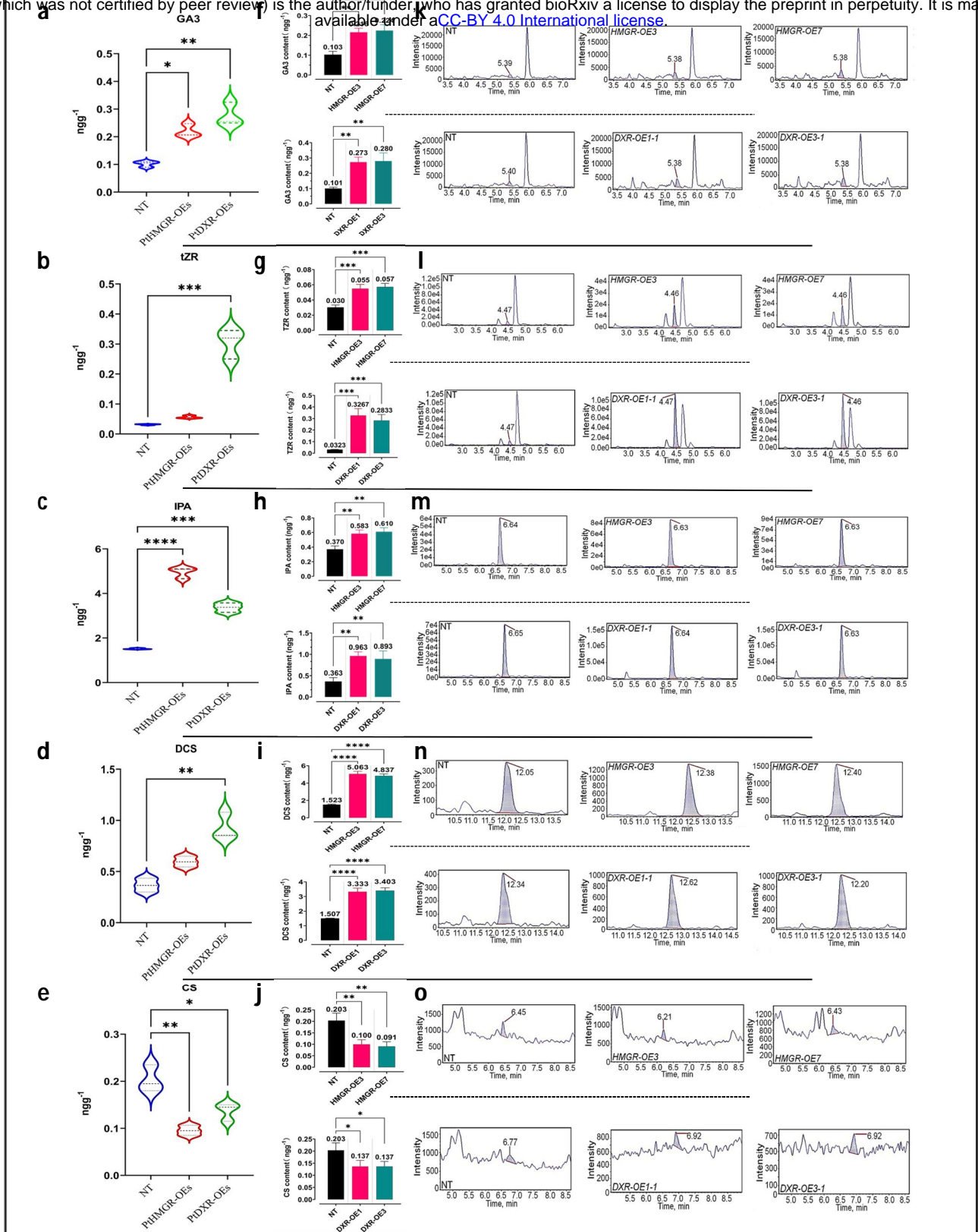


Figure 4 | HPLC-MS/MS content analyses of MEP- and MVA-derived isoprenoids. a,b,c,d, and e, Violin plots reveal the contents of isoprenoids GA3, tZR, IPA, DCS, and CS obtained from MEP- and MVA-pathways influenced by *PthMGR*- and *PtdXR*-OEs. **f,g,h,i, and j,** the column plots reveal the effect of *PthMGR-OE3* and *-7* and *PtdXR-OE1* and *-3* on the mentioned above isoprenoids separately; NT poplars have been used as the control. Bars represent mean \pm SD (n = 3); Stars reveal significant differences, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **k,l,m,n, and o,** represent the HPLC-MS/MS chromatogram content analyses of GA3, tZR, IPA, DCS, and CS, respectively affected by *PthMGR*- and *PtdXR*-OEs comparing with NT poplars.

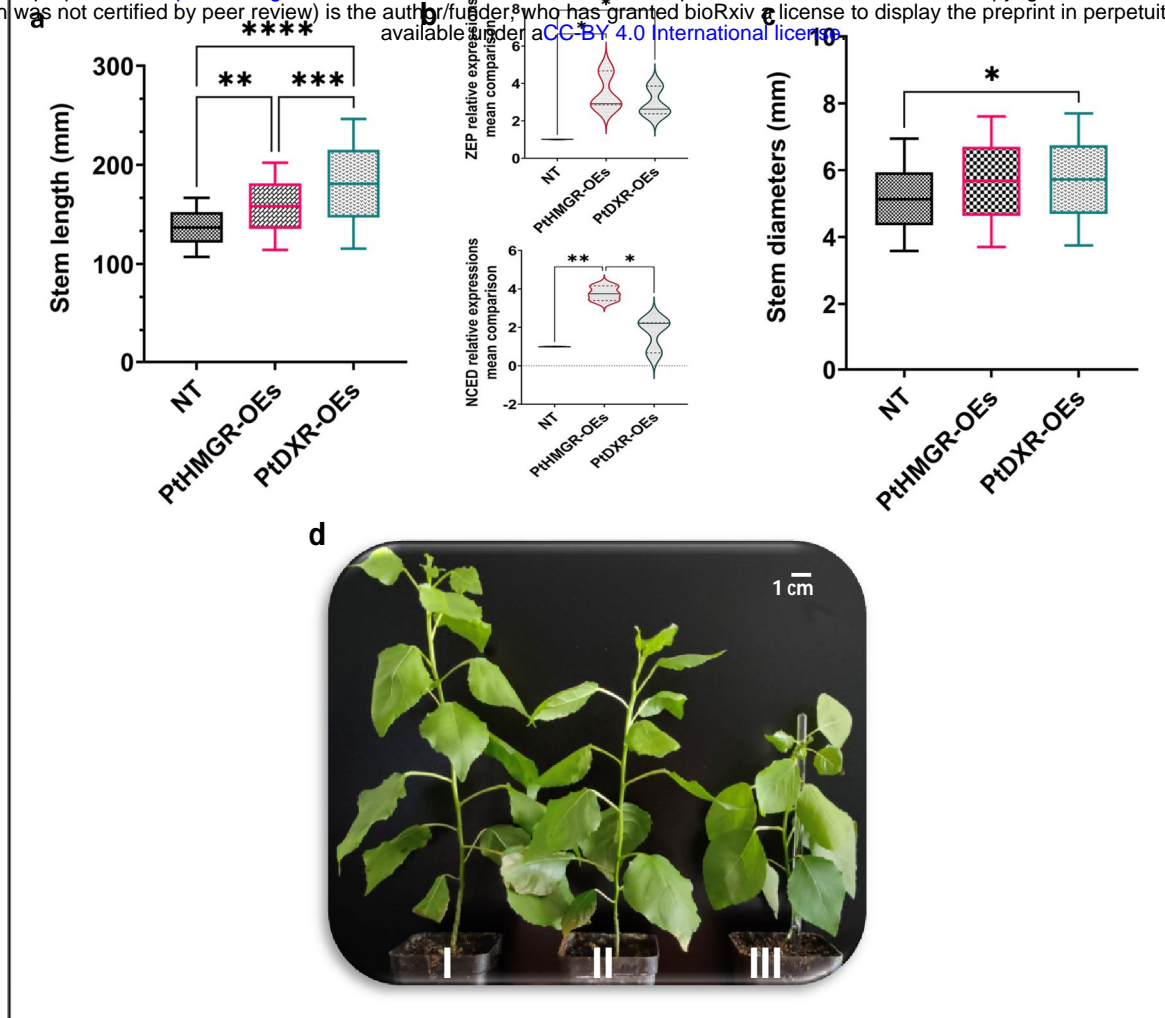


Figure 5 | Phenotypic changes resulted by affected MVA- and MEP- pathway interactions in 45-day-old poplars. **a**, Mean comparisons of stem lengths revealed significantly higher lengths *PtDXR-OEs* than NT poplars compared with *PtHMGR-OEs*. *PtHMGR* transgenics also revealed significantly higher lengths than NT poplars. **b**, Mean comparisons of *ZEP* and *NCED* relative expressions between *PtHMGR*- and *PtDXR-OEs* compared to NT poplars. **c**, Mean comparisons of stem diameters revealed less significant differences between *PtDXR-OEs* and NT poplars. Stars reveal significant differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **d**(I), The *PtDXR* transgenic revealed a higher stem length than *PtHMGR-OEs* and NT poplars. **d**(II), The *PtHMGR* transgenic presents an insignificantly more stem development than NT poplar. **d**(III), NT poplar was used as a control; Scale bar represents 1 cm.

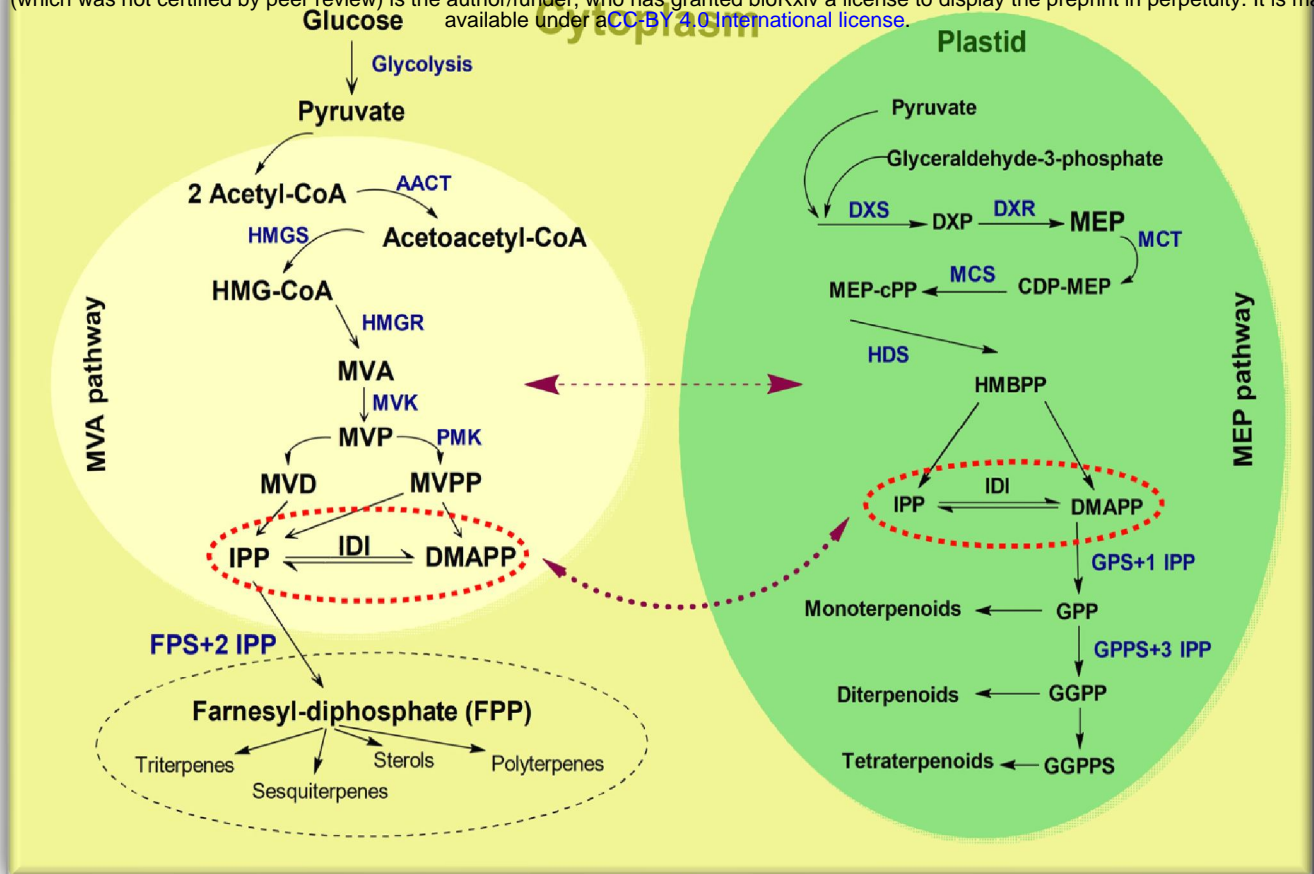
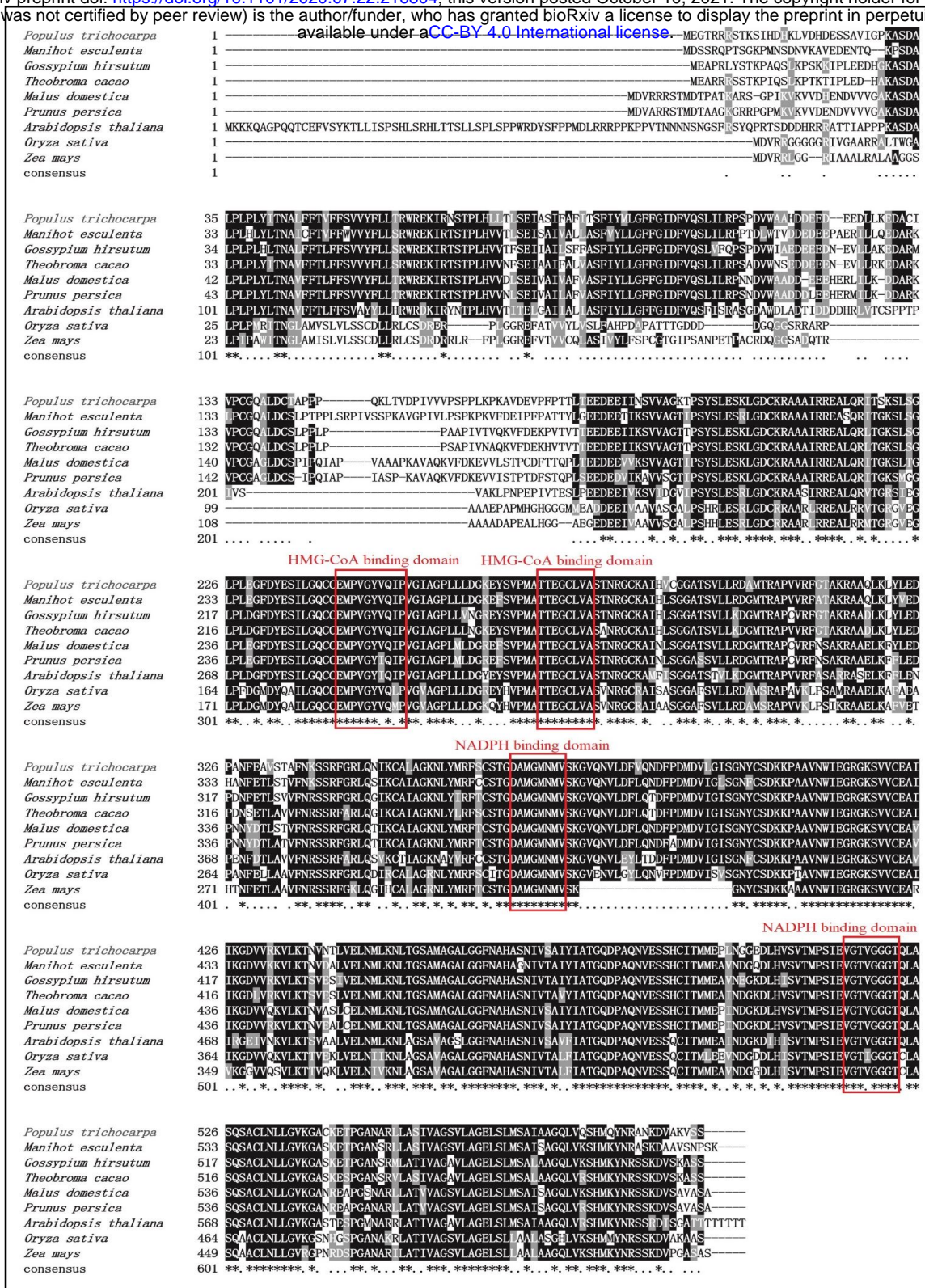
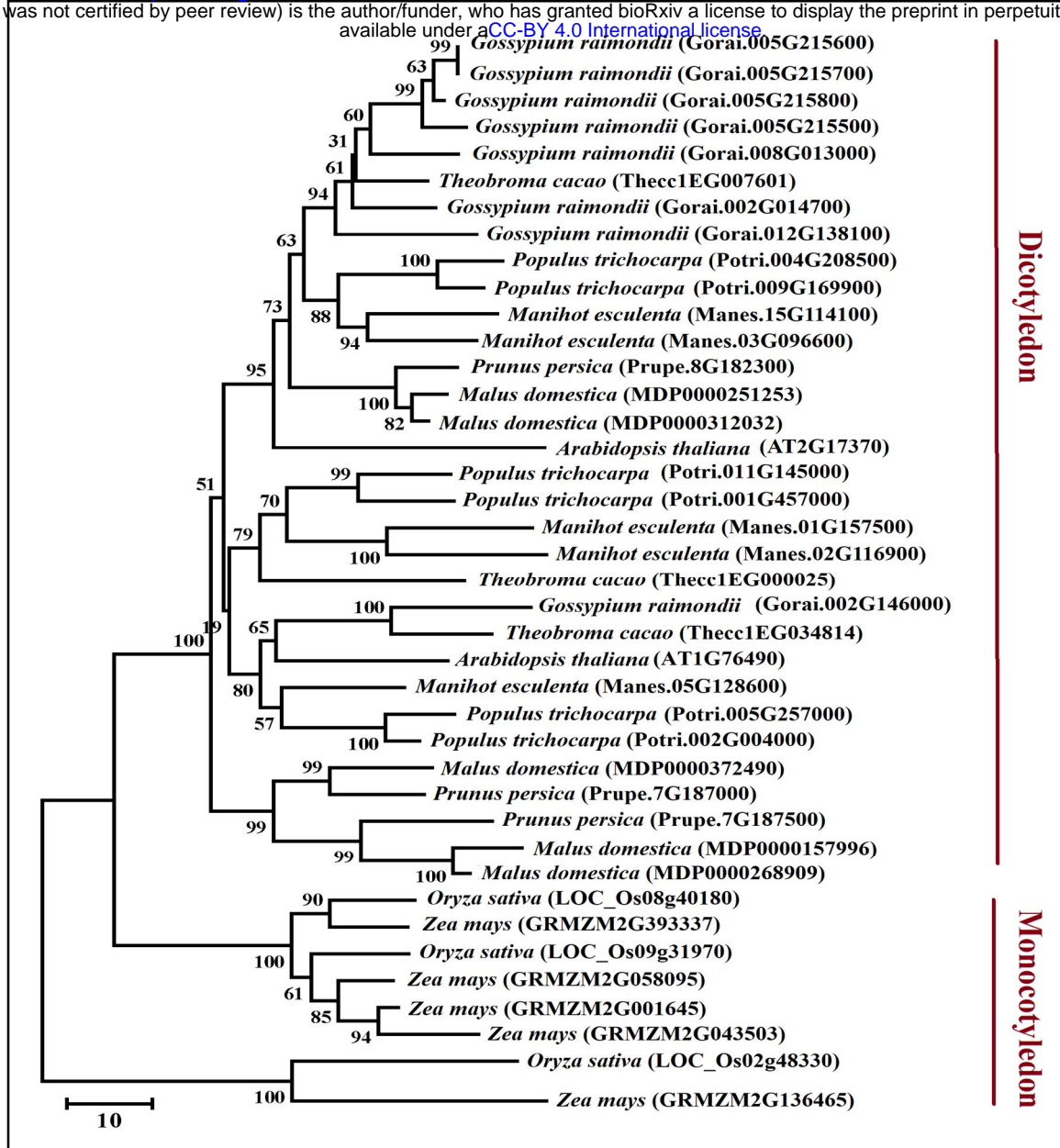


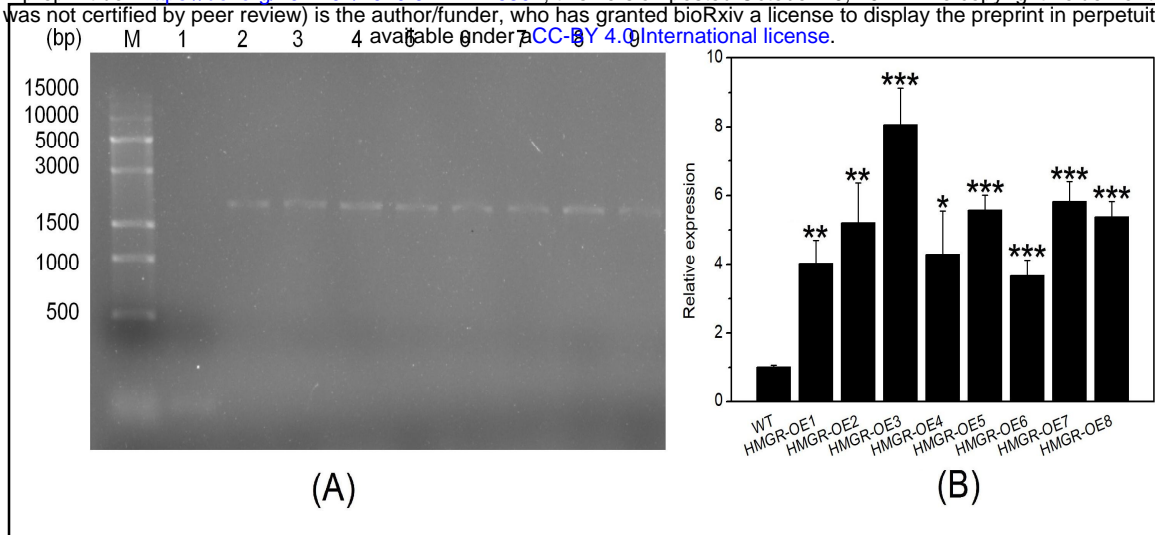
Figure 6 | The interactions between MEP- and MVA-pathways. The IPP and DMAPP are considered the common precursors of the MEP- and MVA-pathway between cytoplasm and plastid. In addition, the putative communication generates between MVA- and MEP-related genes and MVA- and MEP-derived products. MVA: mevalonic acid, MEP: methylerythritol phosphate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, AACT: acetoacetyl CoA thiolase, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, MVK: mevalonate kinase, MVD: mevalonate5-diphosphate decarboxylase, IPP: isopentenyl diphosphate, IDI: IPP isomerase, GPP: geranyldiphosphate, FPP: famesyldiphosphate, GPS: geranyl phosphate synthase, FPS: farnesyl-diphosphate synthase, GPPS: geranyl diphosphate synthase, GGPPS: geranyl geranyl diphosphate synthase, DXS: 1-deoxy-D-xylulose5-phosphate synthase, DXP: 1-deoxy-D-xylulose5-phosphate, DXR: 1-deoxy-D-xylulose5-phosphate reductoisomerase, HDS: 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphate synthase, HDR: 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphate reductase, MCT: MEP cytidyltransferase, CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase.



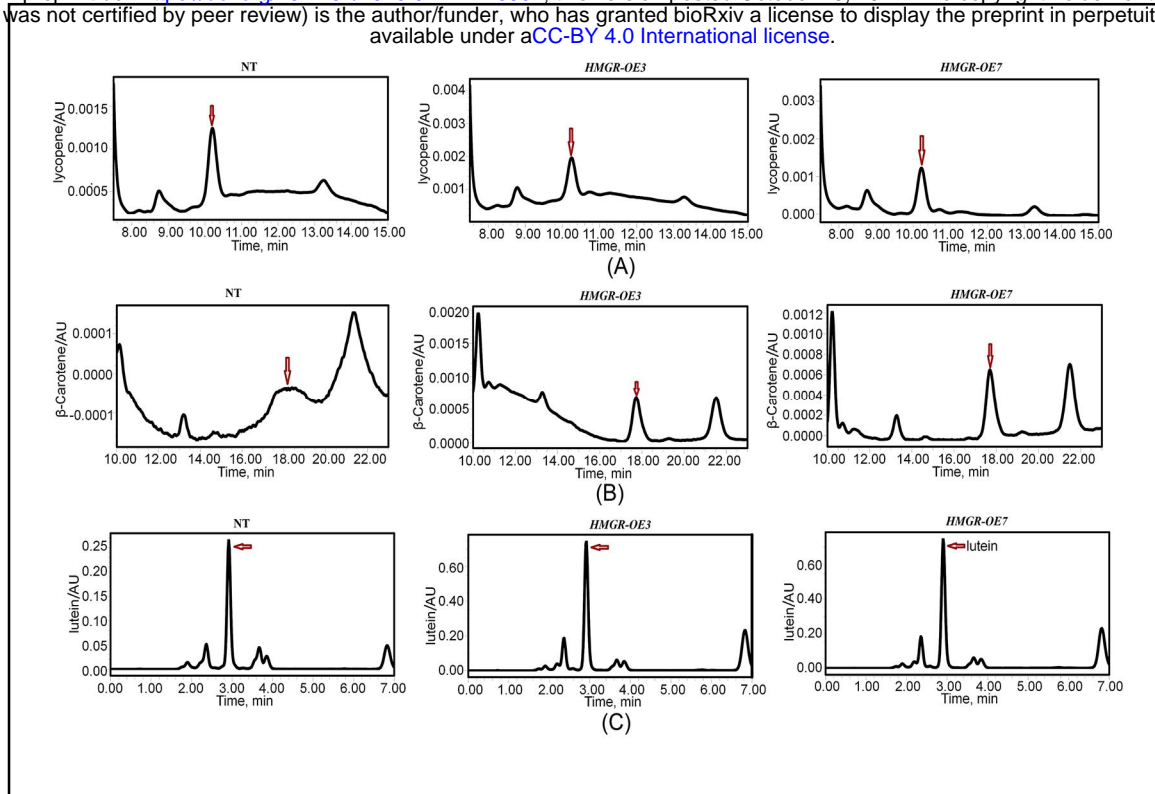
Supplemental Figure 1 | Amino acid sequences alignment of PthMGR protein and other known HMGR proteins. A. *thaliana* (NP_177775.2), *G. hirsutum* (XP_016691783.1), *M. domestica* (XP_008348952.1), *M. esculenta* (XP_021608133.1), *P. persica* (XM_020569919.1), *O. sativa* (XM_015768351.2), *T. cacao* (XM_007043046.2), *Z. mays* (PWZ28886.1). The HMG-CoA and NADPH binding domains are indicated in red rectangle.



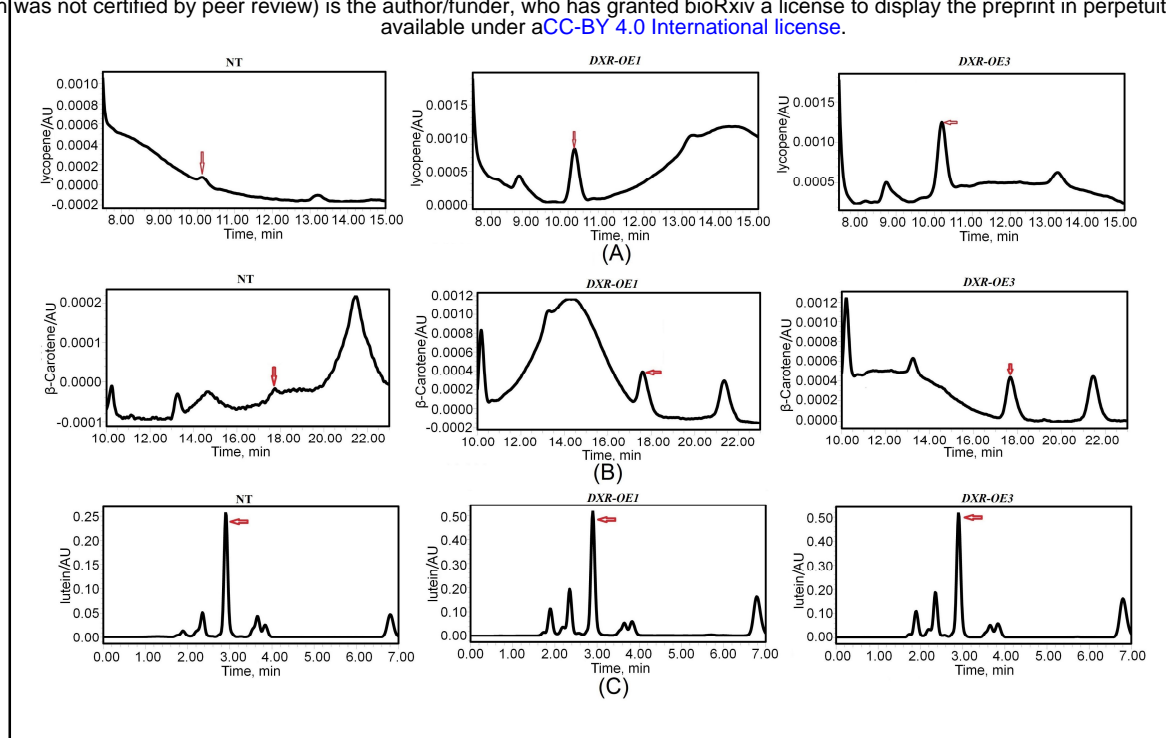
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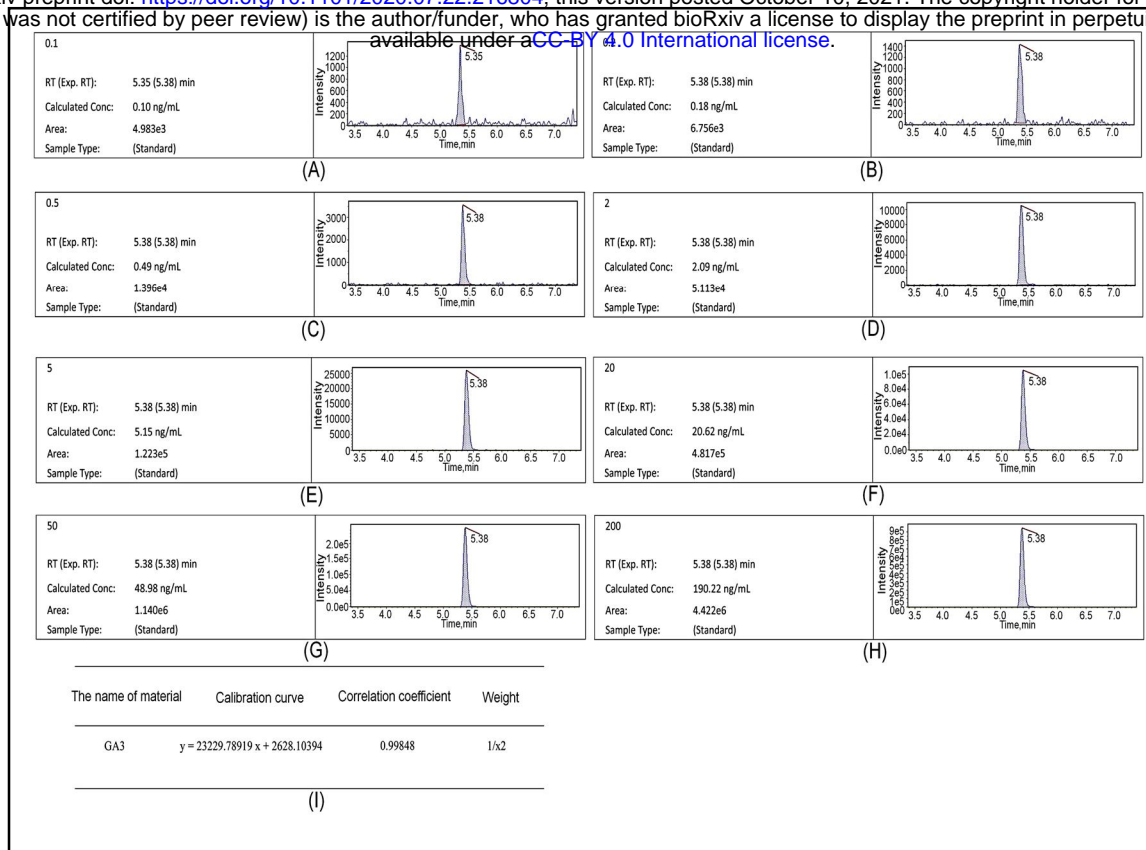
Supplemental Figure 3 | Molecular identification of *PtHMGR-OEs*. (A) PCR identification of *PtHMGR* in *PtHMGR-OEs* and NT poplars. Lane M: 15K molecular mass marker (TransGen, China); lane 1: genomic DNA from WT as a negative control; lanes 2–9: genomic DNAs from *PtHMGR-OE* lines. (B) qRT-PCR identification of the transcript levels of *PtHMGR* in *PtHMGR-OEs* and NT poplars. Three independent experiments were performed; Stars reveal significant differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



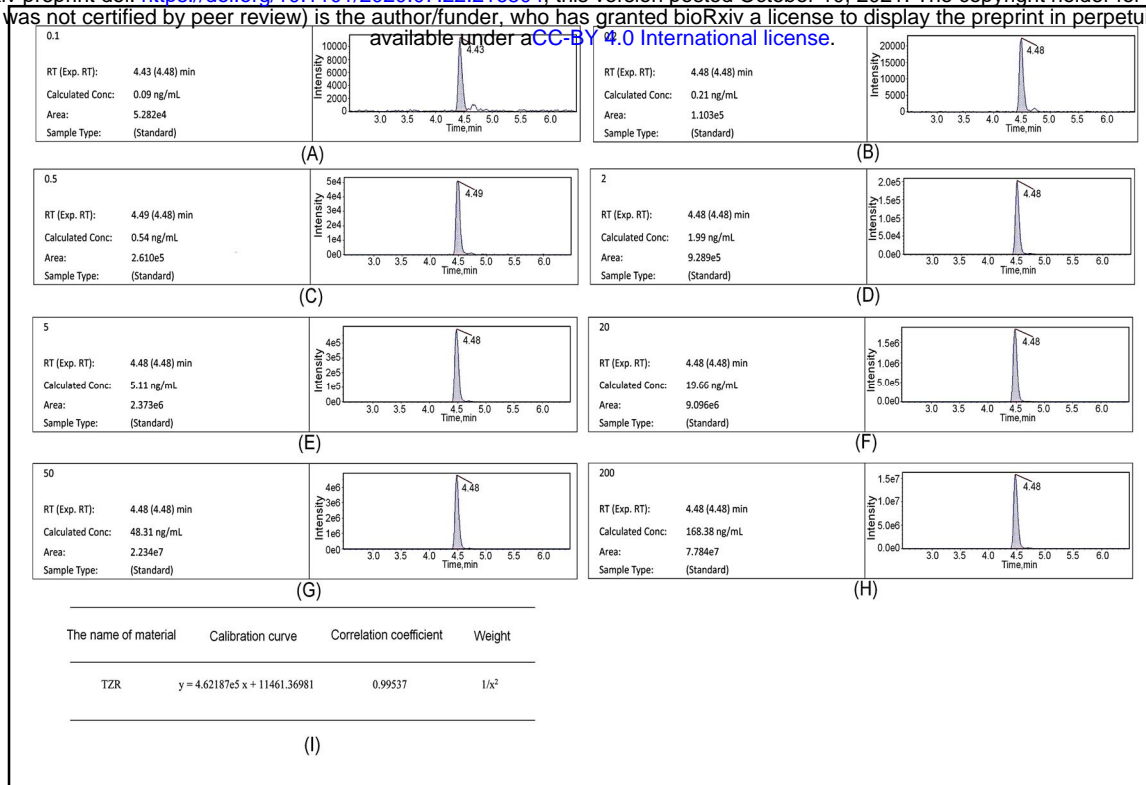
Supplemental Figure 4 | HPLC chromatograms of analyzing the contents of (A) β -carotene, (B) lycopene, and (C) lutein in NT poplars and *PtHMGR-OEs*.



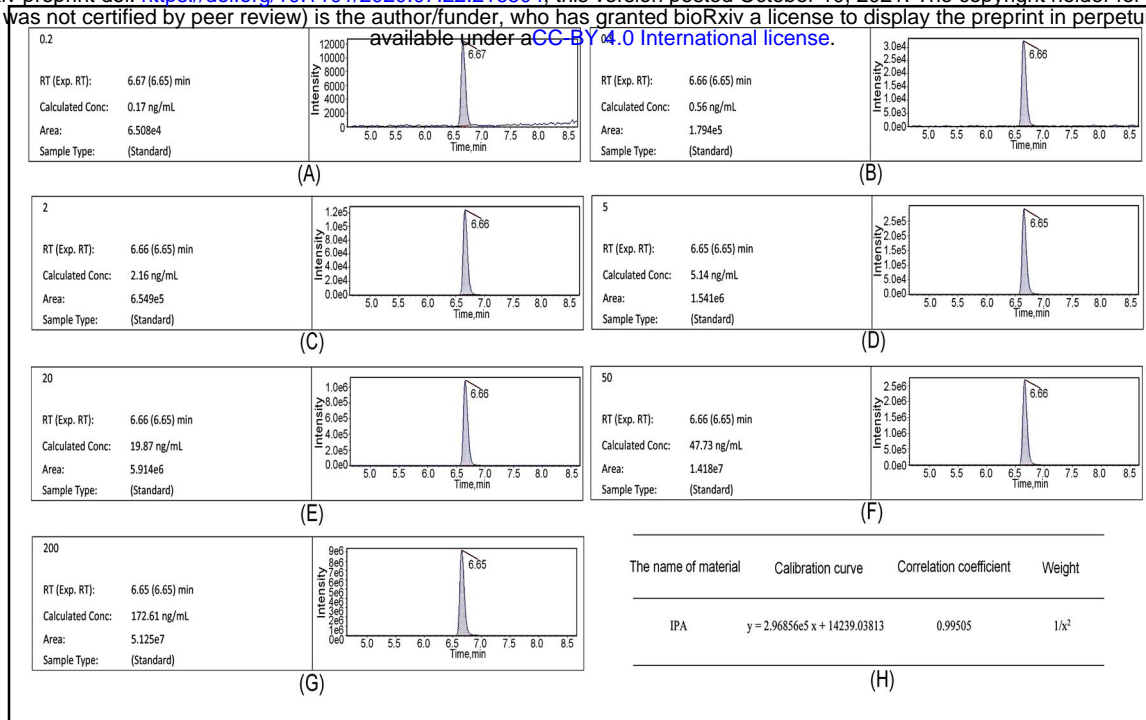
Supplemental Figure 5 | HPLC chromatograms of analyzing the contents of (A) β -carotene, (B) lycopene, and (C) lutein in NT poplars and *PtDXR-OEs*.



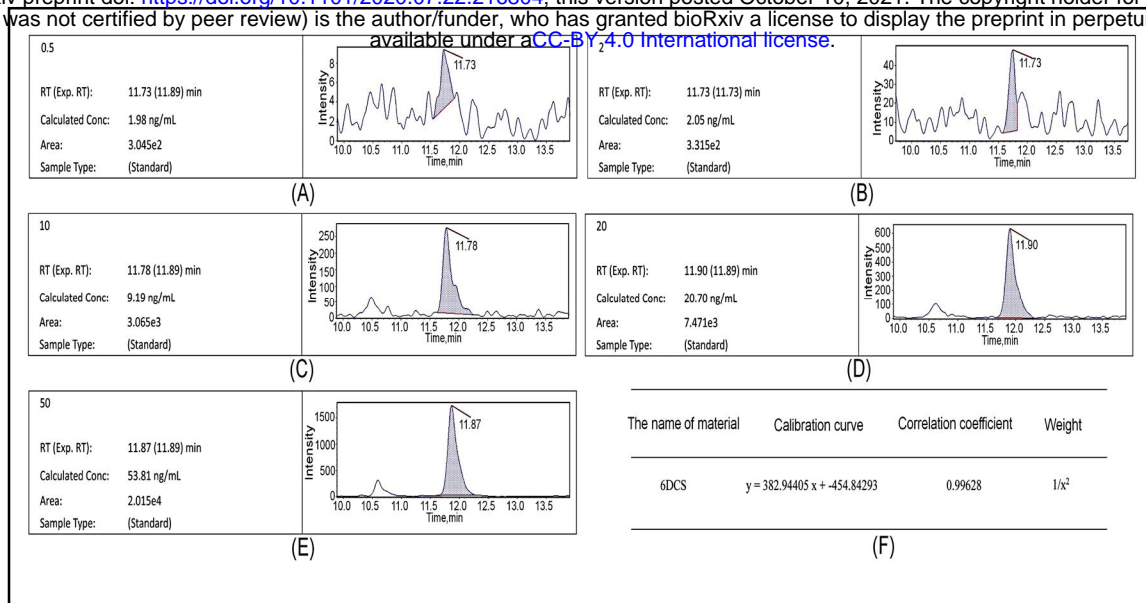
Supplemental Figure 6 | Chromatogram analyses of GA3 standards via HPLC-MS/MS. The chromatogram of standard GA3 at (A) 0.1, (B) 0.2, (C) 0.5, (D) 2, (E) 5, (F) 20, (G) 50, and (H) 200 ng/mL concentrations. (I) Equations for the GA3 standard curves.



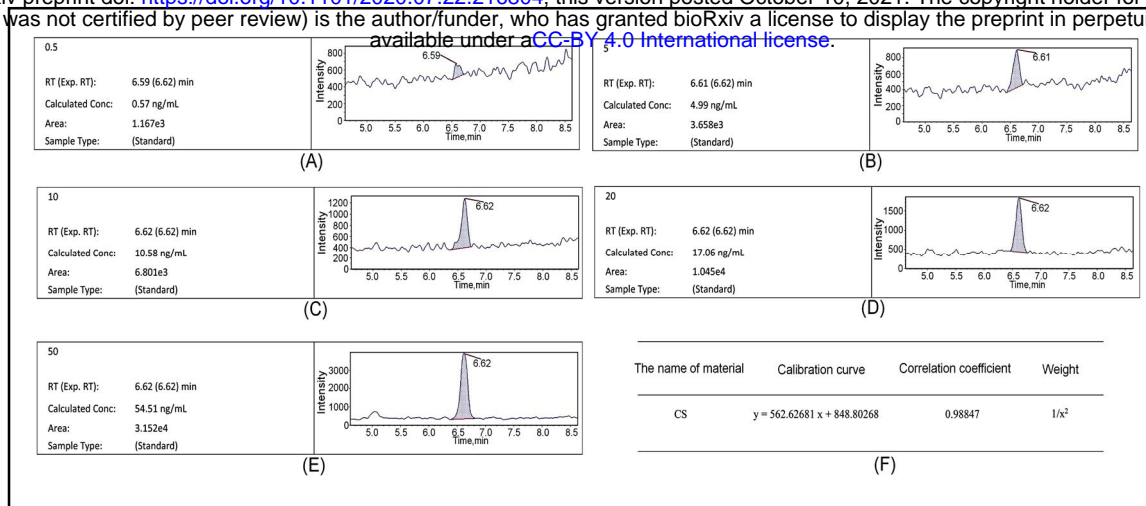
Supplemental Figure 7 | Chromatogram analyses of tZR standards via HPLC-MS/MS. The chromatogram of standard tZR at (A) 0.1, (B) 0.2, (C) 0.5, (D) 2, (E) 5, (F) 20, (G) 50, and (H) 200 ng/mL concentrations. (I) Equations for the TZR standard curves.



Supplemental Figure 8 | Chromatogram analyses of IPA standards via HPLC-MS/MS. The chromatogram of standard IPA at (A) 0.2, (B) 0.5, (C) 2, (D) 5, (E) 20, (F) 50, and (G) 200 ng/mL concentrations. (H) Equations for the IPA standard curves.



Supplemental Figure 9 | Chromatogram analyses of DCS standards via HPLC-MS/MS. The chromatogram of standard DCS at (A) 0.5, (B) 2, (C) 10, (D) 20, and (E) 50 ng/mL concentrations. (F) Equations for the DCS standard curves.



Supplemental Figure 10 | Chromatogram analyses of CS standards via HPLC-MS/MS. The chromatogram of standard CS at (A) 0.5, (B) 5, (C) 10, (D) 20, and (E) 50 ng/mL concentrations. (F) Equations for the CS standard curves.