Isoprenoid biosynthesis regulation in poplars by methylerythritol phosphate and mevalonic acid pathways

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Abstract

The isoprenoids found in plants are extremely important to survive with various human applications, such as flavoring, fragrance, dye, pharmaceuticals, and biomass used for biofuels. Methylerythritol phosphate (MEP) and mevalonic acid (MVA) pathways are critical in plants, responsible for isoprenoid biosynthesis. 1-deoxy-D-xylulose5-phosphate synthase (DXS) and 1-deoxy-D-xylulose5-phosphate reductoisomerase (DXR) catalyze the rate-limiting steps in the MEP pathway, while 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the rate-limiting step in the MVA pathway. Here, we showed while *PtHMGR* overexpressors (OEs) exhibited different MEP- and MVA-related gene expressions compared with non-transgenic poplars (NT), the *PtDXR-OEs* revealed upregulated MEP-related and downregulated MVA-related gene expressions. *PtDXR* and *PtHMGR o*verexpressions caused changes in MVA-derived trans-zeatin-riboside, isopentenyl adenosine, castasterone, and 6-deoxocastasterone well as MEP-derived carotenoids and gibberellins. In *PtHMGR*-OEs, the accumulated geranyl diphosphate synthase (*GPS*) and geranyl pyrophosphate synthase (*GPPS)* transcript levels in the MEP pathway led to an accumulation of MEP-derived isoprenoids. In contrast, upregulation of farnesyl diphosphate synthase (*FPS*) expression in the MVA pathway contributed to increased levels of MVA-derived isoprenoids. In addition, *PtHMGR*-OEs increased MEP-related *GPS* and *GPPS* transcript levels, expanded MEP-derived isoprenoid levels, changed *FPS* transcript levels, and affected MVA-derived isoprenoid yields. These results demonstrate the contribution of MVA and MEP pathways regulating isoprenoid biosynthesis in poplars.

Keywords: MEP; MVA; HMGR; DXR; Isoprenoid biosynthesis

63 ## 1. Introduction

64 Biosynthesis of isoprenoids (terpenoids) is essential for all living organisms. There are
65 over 50,000 distinct molecules in living organisms that are isoprenoids, presenting many
66 functional and structural properties (Thulasiram et al., 2007). Isoprenoids play vital roles in
67 plant growth and development, as well as in membrane fluidity, photosynthesis, and
68 respiration. As specific metabolites, they join in plant-pathogen and allelopathic interactions
69 to preserve plants upon pathogens and herbivores, and they are also created to draw
70 pollinators and seed-dispersing animals. Numerous isoprenoids are of commercial importance
71 for rubber products and drugs, flavors, fragrances, agrochemicals, nutraceuticals,
72 disinfectants, and pigments (Bohlmann and Keeling, 2008). A wide range of isoprenoid
73 biochemical processes are involved in photosynthesis in plants, including electron transfer,
74 quenching of excited chlorophyll triplets, light-harvesting, and energy conversion (Malkin R,
75 2000). Chlorophylls, consisting of the heme pathway-derived tetrapyrrole ring with an
76 appended isoprenoid-derived phytol chain, exist in all reaction center and antenna complexes
77 to absorb light energy and transfer electrons to the reaction centers. The linear or partially
78 cyclized carotenes and their oxygenated derivative xanthophyll are isoprenoids that extinguish
79 excess excitation energy through light-harvesting to preserve the light-harvesting system.
80 However, these isoprenoids operate as attractants in flowers and fruits as well (Rodriguez-
81 Concepcion, 2010). A large proportion of the isoprenoid flux in plants is conducted toward the
82 synthesis of membrane sterol lipids. In contrast to vertebrates synthesizing cholesterol, higher
83 plants synthesize a complex mix of sterol lipids called phytosterols (Boutte and Grebe, 2009).

84 Plants isoprenoids include gibberellins (GAs), carotene, Lycopene, cytokinins (CKs),
85 strigolactones (GRs), and brassinosteroids (BRs) are produced through methylerythritol
86 phosphate (MEP) and mevalonic acid (MVA) pathways (Henry et al., 2015; van Schie et al.,
87 2006; Xie et al., 2008). The mentioned pathways are involved in plant growth, development,
88 and response to environmental changes (Bouvier et al., 2005; Kirby and Keasling, 2009). The
89 isopentenyl diphosphate isomerase (IDI) catalyzes the conversion of the isopentenyl
90 diphosphate (IPP) into dimethylallyl diphosphate (DMAPP), leading to provide the basic
91 materials for all isoprenoid productions (Hemmerlin, 2012; Lu et al., 2012; Zhang et al., 2019).
92 The produced IPP and DMAPP play essential roles in MEP and MVA pathways crosstalk

93 (Huchelmann et al., 2014; Liao et al., 2016). The MVA pathway reactions appear in the
94 cytoplasm, endoplasmic reticulum (ER), and peroxisomes (Cowan et al., 1997; Roberts, 2007),
95 producing sesquiterpenoids and sterols. The 3-hydroxy-3-methylglutaryl-CoA reductase
96 (HMGR), a rate-limiting enzyme in the MVA pathway, catalyzes 3-hydroxy-3-methylglutary-
97 CoA (HMG-CoA) to form MVA (Cowan et al., 1997; Roberts, 2007).

98 Reactions of the MEP pathway occur in the chloroplast and produce carotenoids, GAs,
99 and diterpenoids. 1-deoxy-D-xylulose5-phosphate synthase (DXS) and 1-deoxy-D-xylulose5-
100 phosphate reductoisomerase (DXR) are rate-limiting enzymes in the MEP pathway that
101 catalyze the conversion of D-glyceraldehyde3-phosphate (D-3-P) and pyruvate into 2-C-
102 methyl-D-erythritol4-phosphate (MEP) (Cordoba et al., 2009; Perreca et al., 2020; Wang et al.,
103 2012; Yamaguchi, 2018). Terpenoids like phytoalexin and volatile oils play essential roles in
104 plant growth, development, and disease resistance (Hain et al., 1993; Ren et al., 2008).
105 Photosynthetic pigments convert organic carbon into plant biomass (Esteban et al., 2015). In
106 addition to an extensive range of natural functions in plants, terpenoids also consider the
107 potential for biomedical applications. Paclitaxel is one of the most effective chemotherapy
108 agents for cancer treatment, and artemisinin is an anti-malarial drug (Kim et al., 2016a; Kong
109 and Tan, 2015).

110 Previous metabolic engineering studies have proposed strategies to improve the
111 production of specific metabolites in plants (Ghirardo et al., 2014; Opitz et al., 2014). For
112 example, PMT and H6H encoding the putrescine N-methyltransferase and hyoscyamine 6 β-
113 hydroxylase respectively produced significantly higher scopolamine in transgenic henbane
114 hairy root. Also, HCHL encoding p-hydroxycinnamoyl-CoA hydratase/lyase accumulated the
115 glucose ester of p-hydroxybenzoic acid (pHBA) in Beta vulgaris hairy root (Rahman et al., 2009;
116 Zhang et al., 2004). The 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) is the
117 second enzyme in the MVA pathway. Liao et al. (2018) confirmed that *HMGS* overexpression
118 of *Brassica juncea* upregulates carotenoid and phytosterol in tomatoes. HMGR has been
119 considered a critical factor in metabolically engineering terpenoids (Aharoni et al., 2005;
120 Dueber et al., 2009). In addition, *PgHMGR1* overexpression of ginseng increases ginsenosides
121 content, a necessary pharmaceutically active component (Kim, 2014).

122 Transgenic tobacco overexpressing the *Hevea brasiliensis HMGR* enhanced the
123 phytosterol levels (Schaller et al., 1995). It has been shown (Dai et al., 2011)

124 that *SmHMGR2* in *Salvia miltiorrhiza*, resulting in the improvement of squalene and
125 tanshinone contents. Moreover, *Arabidopsis thaliana HMGR1 (AtHMGR1)* enhanced the
126 phytosterol levels in the first generation of transgenic tomatoes (Enfissi et al., 2005). While
127 the deaccumulation of *DXR* transcripts resulted in lower pigmentation and chloroplast
128 appearance defects, the upregulated *DXR* expression caused the MEP-derived plastid
129 isoprenoids to accumulate. Therefore, *DXR* can be genetically engineered to regulate the
130 content of terpenoids and expressed *DXR* in *Arabidopsis* and observed enhanced flux through
131 the MEP pathway (Carretero-Paulet et al., 2006). While the *A. thaliana DXR* overexpression
132 caused the diterpene anthiolimine to accumulate in *Salvia sclarea* hairy roots (Vaccaro et al.,
133 2014), the peppermint *DXR* overexpression resulted in essential oil inflation (about 50%) with
134 no significant variations in monoterpene composition (Mahmoud and Croteau, 2001).
135 Furthermore, previous studies have shown the exchange of metabolic intermediates included
136 in the MVA- and MEP pathways through plastid membranes (Laule, 2003; Liao, 2006). In
137 summary, the overexpression of genes involved in the MVA- and MEP pathways can change
138 the abundances or activities of related enzymes and metabolic products, causing a new
139 opportunity for plant breeding to enhance the accumulation of related metabolic products.
140 Poplars as an economic and energy species are widely used in industrial and agricultural
141 production. Its fast growth characteristics and advanced resources in artificial afforestation
142 play a vital role in the global ecosystem (Devappa, 2015).
143 This study investigates the poplar isoprenoid biosynthesis. We showed that the
144 overexpression of the MVA-specific *PtHMGR* gene upregulated not only MVA- but MEP-
145 related genes in the transcript levels. We also proved that the overexpression of the MEP-
146 specific *PtDXR* gene caused downregulating MVA-related genes compared with upregulating
147 MEP-related genes, enhancing terpenoid accumulation. Taken together, these results indicate
148 that the MEP is a dominant pathway in contribution with the MVA pathway to produce
149 isoprenoids secondary metabolites, and *HMGR* and *DXR* genes play key regulation points in
150 these pathways.

151 ## 2. Materials and Methods

152 ### 2.1. *Plant materials and growth conditions*

153 Non-transgenic *P. trichocarpa* and *Populus* × *euramericana* cv. 'Nanlin 895' plants were

154 cultured in half-strength Murashige and Skoog (1/2 MS) medium (pH 5.8) under conditions of
155 24°C and 74% humidity (Movahedi et al., 2015). Subsequently, NT and transgenic poplars were
156 cultured in 1/2 MS under long-day conditions (16 h light/8 h dark) at 24°C for 1 month
157 (Movahedi et al., 2018).

158 ## 2.2. *PtHMGR and PtDXR genes isolation and vector construction*

159 To produce cDNA, total RNA was extracted from *P. trichocarpa* leaves and processed with
160 PrimeScript™ RT Master Mix, a kind of reverse transcriptase (TaKaRa, Japan). Forward and
161 reverse primers (Supplementary Table 1: *PtHMGR*-F and *PtHMGR*-R) were designed, and the
162 open reading frame (ORF) of *PtHMGR* was amplified via PCR. We then used the total volume
163 of 50µl including 2 µl primers, 2.0 µl cDNA, 5.0 µl 10 × PCR buffer (Mg2+), 4µl dNTPs (2.5 mM),
164 0.5 µl rTaq polymerase (TaKaRa, Japan) for the following PCR reactions: 95°C for 7 min, 35
165 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,
166 the product of the *PtHMGR* gene was ligated into the pEASY-T3 vector (TransGen Biotech,
167 China) based on blue-white spot screening, and the *PtHMGR* gene was inserted into the vector
168 pGWB9 (Song et al., 2016) using Gateway technology (Invitrogen, USA). On the other hand, all
169 steps to generate cDNA, RNA extraction, PCR, pEASY-T3 ligation, and vector construction
170 (pGWB9-PtDXR) of *PtDXR* have been carried out according to Xu et al. (2019).

171 ## 2.3. *phylogenetic analyses*

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

179 ## 2.4. *Transgenic poplars: generation and confirmation*

180 *Agrobacterium tumefaciens* var. EHA105 was used for the infection of poplar leaves and
181 petioles (Movahedi et al., 2014). Poplar buds were screened on differentiation MS medium

182 supplemented with 30 μg/mL Kanamycin (Kan). Resistant buds were planted in bud elongation
183 MS medium containing 20 μg/mL Kan and transplanted into 1/2 MS medium including 10
184 μg/mL Kan to generate resistant poplar trees. Genomic DNA has been extracted from putative
185 transformants one-month-old leaves grown on a kanamycin-containing medium using
186 TianGen kits (TianGen BioTech, China). The quality of the extracted genomic DNA (250–350
187 ng/μl) was determined by a BioDrop spectrophotometer (UK). PCR was carried out using
188 designed primers (Supplementary Table 1: CaMV35S as the forward and PtHMGR as the
189 reverse), Easy Taq polymerase (TransGene Biotech), and 50 ng of extracted genomic DNA as a
190 template to amplify about 2000 bp. In addition, total RNA was extracted from these one-
191 month-old leaves to produce cDNA, as mentioned above. These cDNA then were applied to
192 reverse transcription-quantitative PCR (RT-qPCR) (Supplementary Table 1: PtHMGR forward
193 and reverse) for comparing the transformants *PtHMGR-OEs* expressions with NT poplars and
194 transforming confirmation.

195 ## 2.5. *Phenotypic properties evaluation*

196 To evaluate phenotypic changes, we selected 45-day-old poplars from PtHMGR-and
197 PtDXR-OEs and NT poplars. We then simultaneously calculated the stem lengths (mm) and
198 stem diameters (mm) every day and recorded them. All recorded were analyzed by GraphPad
199 Prism 9, applying ANOVA one way (Supplementary Table 2).

200 ## 2.6. *Analyses via qRT-PCR*

201 12-month-old *PtDXR-OEs* (Xu et al., 2019) and *PtHMGR-OE* poplars (Soil-grown poplars)
202 have been used to extract total RNA. The qRT-PCR was performed to identify MVA- and MEP-
203 related gene expression levels in NT, *PtDXR-OE*, and *PtHMGR-OE* poplars. The qRT-PCR was
204 served with a StepOne Plus Real-time PCR System (Applied Biosystems, USA) and SYBR Green
205 Master Mix (Roche, Germany). Poplar *Actin* (*PtActin*) (XM-006370951.1) was previously tested
206 as a reference gene for this experiment (Zhang et al., 2013). The following conditions were
207 used for qRT-PCR reactions: pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at
208 95°C for 15 s, and a chain extension at 60°C for 1 min. Three independent experiments were
209 conducted using gene-specific primers (Supplementary Table 1: PtHMGR forward and reverse).

210 2.7. *Metabolite analyses via high-performance liquid chromatography-tandem*
211 *mass spectrometry*

212 The isopropanol/acetic acid extraction method extracted poplar endogenous hormones
213 from NT, *PtDXR-OE*, and *PtHMGR-OE* leaves. GAs and CKs were extracted from, and then HPLC-
214 MS/MS (Qtrap6500, Agilent, USA) was used to quantify levels of GAs, zeatin, tZR, and IPA. Also,
215 methanol considered as solvent was used to extract 5-Deoxystrigol (5-DS), CS, and DCS, and
216 HPLC-MS/MS (Aglient1290, AB; SCIEX-6500Qtrap, Agilent; USA) was also used to determine
217 the contents of 5-DS, CS, and DCS. In addition, acetone, as a solvent, was used to isolate the
218 carotenoid component of poplar leaves. To identify the carotenoid contents, the peak areas
219 of carotenoids analyzed by HPLC (Symmetry Shield RP18, Waters, USA) were used to draw
220 standard carotenoid curves, including β-carotene, Lycopene, and Lutein. Also, the HPLC was
221 used to determine the contents of carotenoids, including β-carotene, Lycopene, and Lutein in
222 NT and OE lines.

223 **3. Results**

224 3.1. *Identification, analyses, and Isolation of PtHMGR and PtDXR genes*

225 *Populus trichocarpa* v3.1 (Phytozome genome ID: 444, NCBI taxonomy ID: 3694) has been
226 applied to download 595 amino acids (aa) PtHMGR (Potri.004G208500.1) and the other
227 species' HMGR to align and analyze. High similarity, including lots of conserved aa
228 accompanied by specific similar domains, HMG-CoA-binding motifs (EMPVGYVQIP' and
229 'TTEGCLVA), and NADPH-binding motifs (DAMGMNMV' and 'VGTVGGGT) (Ma et al., 2012)
230 (Supplementary Figure 1), confirmed the PtHMGR protein analytically. Consequently, a
231 phylogenetic tree based on the various species HMGR supported the PtHMGR candidate
232 identification (Supplementary Figure 2). The tblastn was then applied to reveal 2614
233 bp *PtHMGR* located on Chr04:21681480..21684242 with a 1785 bp CDS. After that, the
234 amplified 1857 bp of the *PtHMGR* from *Populus trichocarpa* cDNA confirmed the putative
235 transgenic lines (Supplementary Figure 3a), exhibiting amplicons in PCR identification
236 compared to NT poplar (Supplementary Figure 3b). The transgenic poplars (*PtHMGR-OEs*) also
237 showed enhanced *PtHMGR* expressions than NT (Supplementary Figure 3c), indicating
238 successful overexpression of *PtHMGR* in poplar. The *PtDXR* gene, which has been isolated,
239 sequenced, and analyzed previously by the authors (Xu et al., 2019), was then transferred into

240 poplar genome to generate PtDXR-OEs used in this study.

241 ### 3.2. *PtHMGR- and PtDXR overexpressions regulate MVA-related gene expressions*

242 MVA-related genes *AACT*, *MVK*, *MVD, and FPS,* except *HMGS,* were significantly
243 upregulated in *PtHMGR-OE* transgenics than NT poplars (Supplementary Figure 4a). In
244 contrast, while only *FPS* revealed significant upregulation by *PtDXR-OEs* in transgenics
245 compared with NT, the other MVA-related genes *AACT*, *HMGS*, *HMGR*, and *MVK* were
246 considerably downregulated (Supplementary Figure 4a). However, the mean comparison of
247 MVA-related gene expressions regulated by *HMGR* and *DXR* overexpressing exhibited
248 significant upregulated *MVK* through *PtHMGR-OEs* (Figure 1a). The *HMGS* was significantly
249 downregulated within *PtHMGR*-and *PtDXR-OEs*, and *MVK* was downregulated
250 by *PtDXR* overexpression (Figure 1a). The mean comparison of *AACT*, *MVD*, and *FPS* revealed
251 upregulation through *PtHMGR-OEs*, while *AACT* and *MVD* showed downregulation
252 through *PtDXR-OEs* (Figure 1a).

253 ### 3.3. *PtHMGR- and PtDXR overexpressions regulate MEP-related gene expressions*

254 While, the expression of MEP-related genes *DXS*, *DXR*, 1-hydroxy-2-methyl-2-(E)-butenyl-
255 4-diphosphate synthase (*HDS*), 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase
256 (*HDR*), *IDI*, and *GPPS* were significantly upregulated in all *PtHMGR-OEs* transgenic poplars in
257 comparison with NT, the *GPS* overexpression was enhanced only by *PtHMGR-*
258 *OE3* (Supplementary Figure 4c). In addition, 2-C-methyl-d-erythritol4-phosphate
259 cytidylyltransferase (*MCT*) and 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (*CMK*) have
260 been downregulated by *PtHMGR-OEs* (Supplementary Figure 4c). In contrast, all MEP-related
261 genes were upregulated significantly by *PtDXR* overexpression (Supplementary Figure 4d). In
262 total, the comparison of the effect of *PtHMGR* on MEP-related genes exhibited significant
263 upregulations of *HDR*, *IDI*, *GPS*, and *GPPS* (Figure 1b). These comparisons also revealed the
264 upregulation of *DXS* and *HDS* except *MCT* and *CMK*, downregulated by *PtHMGR*
265 overexpression (Figure 1b). In contrast, the mean comparison of the effect of *PtDXR*
266 overexpression on MEP-related genes exhibited upregulation of the all mentioned above
267 genes (Figure 1b).

268 3.4. *MVA- and MEP-derived carotenoids are affected by PtHMGR-and PtDXR-OEs*

269 β-carotene is a carotenoid synthesis that has been broadly used in the industrial
270 composition of pharmaceuticals and as food colorants, animal supplies additives, and
271 nutraceuticals. MVA-and MEP pathways have been proved effective in the biosynthesis of β-
272 carotene (Yang, 2014). In addition, Lycopene is a carotenoid referring to C40 terpenoids and
273 is broadly found in various plants, particularly vegetables and fruits. It has been shown that
274 MVA and MEP-pathways directly influence the biosynthesis production of Lycopene (Kim et
275 al., 2019; Wei et al., 2018). While Wille et al. (2004) showed that β-carotene and Lutein are
276 synthesized using intermediates from the MEP pathway, Opitz et al. (2014) revealed that both
277 MVA and MPE pathways contribute to producing isoprenoids such as β-carotene and Lutein.
278 HPLC-MS/MS has analyzed the quantity of MVA and MEP derivatives. Our analyses revealed
279 that *HMGR*-OEs caused a significant enhancement in Lycopene (an average of ~ 0.08 ug/g), β-
280 carotene (an average of ~ 0.33 ug/g), and Lutein (an average of ~ 272 ug/g) production
281 compared with NT poplars (~0.02, ~0.08, and ~100 ug/g respectively) (Figure 2a, b, and c;
282 Supplementary Figure 5). The ABA-related gene expressions also have been calculated. Results
283 revealed a significantly increased *ZEP1*, *2*, and *3* relative gene expressions with averages of
284 ~2.85, ~4.67, and ~2.92 compared to NT with an average of ~1 (Figure 2d). These results also
285 showed meaningful enhancements of *NCED1, 2,* and *3* relative gene expressions with the
286 averages of ~4.16, ~3.79, and ~3.4 compared to NT with an average of ~1 (Figure 2e).

287 On the other hand, the levels of the MEP-derived substances lycopene, β-carotene, and
288 Lutein were significantly increased in *PtDXR*-OEs with the averages of ~0.08, 0.22, 209.32 ug/g,
289 respectively compared to NT poplars (Figure 3a, b, and c; Supplementary Figure 6). The
290 analyses of ABA-related gene expressions revealed significantly increased *ZEP1*, *2*, and *3*
291 relative gene expressions with the averages of ~2.63, ~2.38, and ~3.86 compared to NT with
292 an average of ~1 (Figure 3d). These results also showed meaningful enhancements of *NCED2*
293 and *3* relative gene expressions with averages of ~2.25 and ~2.21 compared to NT with an
294 average of ~1 (Figure 2e). These results revealed a decreased average in *NCED1* relative gene
295 expression with an average of ~0.66 compared to NT poplars.

296 3.5. *MVA and MEP-related derivatives are influenced by PtHMGR- and PtDXR-OEs*

297 The other MVA and MEP derivatives such as GAs, trans-zeatin-riboside (tZR), isopentenyl

298 adenosine (IPA), 6-deoxyocastasterone (DCS), and castasterone (CS) productions affected
299 by *PtHMGR*- and *PtDXR-OEs* have been analyzed. While Gibberellic acid (GA3) (a downstream
300 product of MEP) (an average of ~0.22 ng/g), tZR (an average of ~0.06 ng/g), IPA (an average
301 of ~0.59 ng/g), DCS (an average of 4.95 ng/g) revealed significantly more productions induced
302 by *HMGR*-OEs, the CS production (~0.095 ng/g) was decreased considerably compared to NT
303 poplars (~0.10, ~0.03, ~0.37, ~1.50, and ~0.20 ng/g respectively) (Figure 4a-j). These results
304 demonstrate that the *HMGR* gene interacts with MVA and MEP derivatives productions in
305 plants. On the other hand, the *PtDXR* overexpression significantly affected the contents of
306 MEP- and MVA-derived products except for CS. *PtDXR*-OEs showed a significant increase
307 ~0.276 ng/g in the GA3 content (Figure 4a and f). The tZR content represented a 10-fold
308 increase (~0.304 ng/g) affected by *PtDXR-OEs* compared to NT poplars (0.032 ng/g) (Figure 4b
309 and g). The content of IPA in *PtDXR-OEs* meaningfully increased ~ 0.928 ng/g, compared to
310 0.363 ng/g in NT poplars (Figure 4c and h) with a 3-fold increase. In addition, the DCS content
311 considerably increased to ~3.36 ng/g, compared with ~1.50 ng/g in NT, representing a 3-fold
312 increase in *PtDXR-OEs* (Figure 4d and i). By contrast, the content of CS in *PtDXR-OEs*
313 significantly decreased (~0.137 ng/g) compared to NT poplar (0.203 ng/g), indicating
314 significant down-regulation in *PtDXR-OEs* (Figure 4e and j). The HPLC-MS/MS chromatograms
315 of GA, tZR, IPA, DCS, and CS standards are provided in Supplementary Figures 7–11.

316 ### 3.6. *Phenotypic properties*

317 To investigate the growth and development resulting from different produced
318 isoprenoids contents amongst the affected MVA-and MEP pathways contributions
319 by *PtHMGR*-and *PtDXR-OEs,* we decided to evaluate phenotypic stem lengths and diameters
320 changes. Results exhibited a significant increase in GA3 contents in *PtDXR-OEs* (Figure 4a)
321 associated with a considerable rise in cytokinin tZR (Figure 4b), resulting in significantly more
322 development in stem length compared to *PtHMGR-OEs* and NT poplars (Figure 5a and b).
323 Regarding increasing ABA-related gene expressions (*ZEP* and *NCED*) in *PtHMGR-OEs* than
324 *PtDXR-OEs* and NT poplars (Figure 5c and d) and also concerning insufficient increase cytokinin
325 tZR in *PtHMGR-OEs* compared with NT poplars (Figure 4b), *PtHMGR* transgenics showed a
326 shorter stem length that *PtDXR* transgenics compared with NT poplars (Figure 5a and b). We
327 also observed that only *PtDXR-OEs* revealed a few significant increases in stem diameters than

328 PtHMGR-OEs and NT poplars (Figure 5e).

329 ## 4. Discussion

330 ### 4.1. *The HMGR and DXR crucial roles in isoprenoid biosynthesis*

331 Several studies report that HMGR activity is regulated by isoprenoid outcomes when
332 stigmasterol and cholesterol reduce the HMGR activity by 35% (Russell and Davidson, 1982).
333 Utilization of the isoprenoid growth control abscisic acid also prevented HMGR activity in pea
334 by about 40%, while zeatin and gibberellin, other isoprenoid growth regulators, improved the
335 activity of HMGR (Russell and Davidson, 1982). Maurey et al. (1986) reported that the
336 alga *Ochromonas malhamensis* developed in mevinolin exhibited to 15-fold increase in
337 microsomal HMGR activity, slightly influencing cell growth. Moreover, the MEP pathway is the
338 primary precursor for required plastid isoprenoids (Wright et al., 2014). It has been shown
339 that volatile compounds made by the MEP pathway are involved in plant protection against
340 biotic and abiotic stresses (Gershenzon and Dudareva, 2007). By modifying the expression of
341 DXR, promising metabolite compounds have developed in the mint plant (Mahmoud and
342 Croteau, 2002). In addition, the DXR overexpression in Arabidopsis resulted in accumulating
343 isoprenoids such as tocopherols, carotenoids, and chlorophylls (Carretero-Paulet et al., 2006).
344 DXR overexpression has also been proven to improve diterpene contents in transgenic
345 bacteria (Morrone et al., 2010). Biotic stresses are vital in providing pharmaceutical
346 terpenoids by expanding the number of enzymes included in biosynthetic pathways by
347 controlling biosynthetic genes expression (Kang et al., 2009; Lu et al., 2016). Biotic stresses
348 caused to improve DXR expression followed by triptophenolide content in *Tripterygium*
349 *wilfordii* cell culture suspension (Tong et al., 2015).

350 ### 4.2. *Overexpression of PtDXR results in upregulation of isoprenoid biosynthesis*
351 *gene expression*

352 Liao et al. (2018) showed that overexpression of *BjHMGS1* affects the expression levels
353 of MEP- and MVA-related genes and slightly increases the transcript levels of *DXS* and *DXR* in
354 transgenic plants. However, *DXS*, *DXR*, *HDS*, and *HDR* expression levels have been
355 upregulated significantly in *PtHMGR-OE* poplars, while *MCT* and *CMK* are downregulated.

356 Similar to Liao et al. (2018) which the *BjHMGS1* overexpression in tomatoes significantly

357 increased the *GPS* and *GPPS* expressions, we exhibited that the *PtHMGR* overexpression
358 enhanced the farnesyl diphosphate synthase (*FPS*), *GPS*, and *GPPS* expressions may
359 stimulate the crosstalk between IPP and DMAPP, increasing the biosynthesis of plastidial C15
360 and C20 isoprenoid precursors. Xu et al. (2012) showed that *HMGR* overexpression
361 in *Ganoderma lucidum* caused upregulated *FPS*, squalene synthase (*SQS*), or lanosterol
362 synthase (LS) mRNA expressions and developed the contents of ganoderic acid and
363 intermediates, including squalene and lanosterol. In addition, the *BjHMGS1* overexpression in
364 tomatoes significantly increased transcript levels of *FPS*, *SQS*, squalene epoxidase (*SQE*), and
365 cycloartenol synthase (*CAS*) (Liao et al., 2018). This study exhibited that except
366 for *HMGS* downregulating, the *AACT*, *MVK*, and *MVD* transcript levels were significantly
367 upregulated in *PtHMGR-OE* poplars. We revealed that these enhanced gene expressions
368 mainly were associated with the MVA-related genes contributing to the biosynthesis of
369 sesquiterpenes and other C15 and universal C20 isoprenoid precursors.

370 ### 4.3. *Overexpression of PtDXR affects MEP- and MVA-related genes*

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

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

383 In this study, while the *PtDXR-OEs* exposed higher MEP-related gene expressions than NT
384 poplars, the *PtDXR-OEs* revealed significant downregulated MVA-related gene expressions
385 than NT poplars. These findings illustrate that the MEP pathway regulates monoterpenes,
386 diterpenes, and tetraterpenoids biosynthesis and could affect the MVA pathway.

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

392 4.4. *Overexpression of HMGR promotes the formation of GAs, and carotenoids in*
393 *plastids and accumulation of tZR, IPA, and DCS in the cytoplasm*

394 HMGR, as the rate-limiting enzyme in the MVA-pathway of plants, plays a critical role in
395 controlling the flow of carbon within this metabolic pathway. The upregulation
396 of *HMGR* significantly increases isoprenoid levels in plants. Overexpression of *HMGRs* of
397 different plant species has been reported to raise isoprenoids levels significantly. The
398 heterologous expression of *Hevea brasiliensis HMGR1* in tobacco increased the sterol content
399 and accumulated intermediate metabolites (Schaller et al., 1995). The *A. thaliana HMGR*
400 *o*verexpression in *Lavandula latifolia* increased the levels of sterols in the MVA-and MEP-
401 derived monoterpenes and sesquiterpenes (Munoz-Bertomeu et al., 2007). In addition,
402 the *Salvia miltiorrhiza SmHMGR* overexpression in hairy roots developed MEP-derived
403 diterpene tanshinone (Kai et al., 2011). In our study, ABA synthesis-related genes
404 (*NCED1*, *NCED3*, *NCED6*, *ZEP1*, *ZEP2*, and *ZEP3*) and the contents of GA3 and carotenoids
405 were upregulated in *PtHMGR-OE* poplar seedlings. This finding suggests that
406 the *HMGR* overexpression may indirectly affect the biosynthesis of MEP-related isoprenoids,
407 including GA3 and carotenoids. The accumulation of MVA-derived isoprenoids including tZR,
408 IPA, and DCS was significantly elevated in *PtHMGR-OEs*, indicating
409 that *PtHMGR* overexpression directly influences the biosynthesis of MVA-related isoprenoids.
410 Therefore, the *HMGR* gene directly affects MVA-derived isoprenoids and indirectly affects the
411 content of MEP-derived isoprenoids by changing the expression levels of MEP-related genes.

412 4.5. *Higher levels of MEP- and MVA-derived products in PtDXR-OE seedlings*

413 DXR is the rate-limiting enzyme in the MEP pathway and an essential regulatory step in
414 the cytoplasmic metabolism of isoprenoid compounds (Takahashi et al., 1998). Mahmoud and
415 Croteau (2001) revealed that overexpression of *DXR* in *Mentha piperita* promoted the
416 synthesis of monoterpenes in the oil glands and increased the production of essential oil yield

417 by 50%. However, the up-regulation of *DXR* expression did not lead to change in the complex
418 oil composition significantly. Hasunuma et al. (2008) exhibited that overexpression of
419 *Synechocystis sp*. strain PCC6803 *DXR* in tobacco resulted in increased levels of β-carotene,
420 chlorophyll, antheraxanthin, and Lutein. Xing et al. (2010) showed that the *A. thaliana dxr*
421 mutants caused to lack of GAs, ABA, and photosynthetic pigments (REF57). These mutants
422 showed pale sepals and yellow inflorescences (Xing et al., 2010). In our study, the relatively
423 higher abundance of GA3 and carotenoids in *PtDXR-OE* poplar seedlings indicated an effect of
424 *DXR* overexpression. Combined with the result described above of increased *DXS*, *HDS*, *HDR*,
425 *MCT*, *CMK*, *FPS*, *GPS*, and *GPPS* expression levels, we postulate that overexpression of *DXR* not
426 only affects the expression levels of MEP-related genes but also changes the field of GA3, and
427 carotenoids.

428 4.6. *Communications exist between MVA- and MEP-pathways excess of IPP and*
429 *DMAPP*

430 Although the substrates of MVA- and MEP pathways differ, there are common
431 intermediates like IPP and DMAPP (Figure 6). Blocking only the MVA or the MEP pathway,
432 respectively, does not entirely prevent the biosynthesis of terpenes in the cytoplasm or
433 plastids, indicating that some MVA and MEP pathways products can be transported and/or
434 move between cell compartments (Aharoni et al., 2003; Aharoni et al., 2004; Gutensohn et
435 al., 2013). For example, it has been shown that the transferring IPP from the chloroplast to
436 cytoplasm observed through 13C labeling, indicating that plentiful IPP is available for use in
437 the MVA-pathway to produce terpenoids (Ma et al., 2017). In addition, segregation between
438 the MVA- and MEP pathways is limited and might exchange some metabolites over the plastid
439 membrane (Laule, 2003). Kim et al. (2016b) used clustered, regularly interspaced short
440 palindromic repeats (CRISPR) technology to reconstruct the lycopene synthesis pathway and
441 control the flow of carbon in the MEP-and MVA-pathways. The results showed that the
442 expression of MVA-related genes was reduced by 81.6%, but the lycopene yield was
443 significantly increased. By analyzing gene expression levels and metabolic outcome
444 in *PtHMGR*-and *PtDXR-OEs*, we discovered that the correlation might exist between MVA- and
445 MEP-related genes with MVA- and MEP-derived products, which are not restricted to crosstalk
446 between IPP and DMAPP (Figure 6).

447 On the one hand, overexpression of *PtDXR* affected the transcript levels of MEP-related
448 genes and the contents of MEP-derived isoprenoids, including GAs and carotenoids. The
449 diminished accumulation of MVA-related gene products causes a reduction in the yields of
450 MVA-derived isoprenoids (including CS) but leads to increasing tZR, IPA, and DCS contents. We
451 hypothesize that IPP and DMAPP produced by the MEP pathway could enter the cytoplasm to
452 compensate for the lack of IPP and DMAPP, and the IPP and DMAPP as the precursors of the
453 MVA pathway are used to guide the synthesis of MVA-derived products. On the other
454 hand, *PtHMGR-OEs* exhibited higher transcript levels of *AACT*, *MVK*, and *MVD* and
455 higher *DXS*, *DXR*, *HDS*, and *HDR* than NT poplars, resulting in effect both MEP- and MVA-
456 related gene expressions. We successfully demonstrated that manipulation of *HMGR* in the
457 poplar MVA pathway results in dramatically enhanced yields of GAs and carotenoids. This
458 result illustrates that cytosolic *HMGR* overexpression expanded plastidial GPP- and GGPP-
459 derived products, such as carotenoids. Therefore, this study provides hints that
460 communications between the MVA-and MEP pathways increased the expression levels
461 of *GPS* and *GPPS* in *PtHMGR-OEs*, and elevated the contents of GA3 and carotenoids.
462 Moreover, changes in MEP- and MVA-related gene expressions affect MVA- and MEP-derived
463 isoprenoids.

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

472 4.7. *Communications between MVA- and MEP pathways affected by PtHMGR- and*
473 *PtDXR-OEs influence the plant growth and developments*

474 It has been shown that Abscisic acid (ABA) and GA3 perform essential functions in cell
475 division, shoot growth, and flower induction (Xing et al., 2016). It has also been demonstrated
476 that the cytokinin tZR, a variety of phytohormones, perform a crucial function as root to shoot

signals, directing numerous developmental and growth processes in shoots (Abul et al., 2010; Sakakibara, 2006). Regarding these findings, we showed how the communications between MVA- and MEP pathways and their changes affected by some stimulators (*HMGR*-and *DXR-OEs*) influenced plant growth, especially in stem length. Finally, We figured out that the gibberellic acid and cytokinin may be more effective in plant growth than inhibiting by ABA, causing higher *PtDXR-OEs* than *PtHMGR-OEs* compared with NT poplars.

Author contributions

A.M. and H.W. conceived, planned, and coordinated the project, performed data analysis, wrote the draft, and finalized the manuscript. B.P. validated and contributed to data analysis and curation, revised and finalized the manuscript. T.J., W.S., and D.L. reviewed and edited the manuscript. L.Y. and Q.Z. coordinated, contributed to data curation, finalized and funded this research. A.M., H.W., and B.P. contributed equally as the first author.

Conflict of interest

The authors declare that they have no conflict of interest.

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697 ## 6. Figure legends

698 **Figure 1 | MVA- and MEP-related genes analyses in overexpressed *PtHMGR*- and *PtDXR-OE*
699 ***s* poplars. a**, Mean comparison of relative expression of MVA-relate genes *AACT*,
700 *HMGS*, *MVK*, *MVD,* and *FPS* (Indicated in red) affected by *PtHMGR* overexpressing. **b**, Mean
701 comparison of relative expression of MEP-related genes *DXS*, *MCT*, *CMK*, *HDS*, *HDR*, *IDI, GPS,*
702 and *GPPS* (Indicated in red) affected by *PtHMGR* overexpressing. *HMGR* and *DXR*, which were
703 overexpressed respectively among *DXR*- and *HMGR-OEs,* were presented in Supplementary
704 Figure 4. *PtActin* was used as an internal reference in all repeats; "ns" means not significant,
705 * P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001; Three independent replications were p
706 erformed in this experiment.

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

714 **Figure 3 | HPLC-MS/MS content analyses of lycopene, β-carotene, Lutein, and real-time PCR
715 of *ZEP* and *NCED* genes family.** HPLC-MS/MS content analyses have been performed to show
716 the effect of *PtDXR-OEs* on **a***,* lycopene **b**, β-carotene, and **c**, lutein. Relative expressions have
717 been analyzed affected by *PtDXR-OEs* compared with NT poplars of **d**, *ZEP*, and **e**, *NCED* genes
718 family. Bars represent mean ± SD (n = 3); Stars reveal significant differences, * P < 0.05, ** P <
719 0.01, *** P < 0.001, ****P < 0.0001; Three independent experiments were performed in these
720 analyses.

721 **Figure 4 | HPLC-MS/MS content analyses of MEP- and MVA-derived isoprenoids. a,b,c,d,
722 and e,** Violin plots reveal the contents of isoprenoids GA3, tZR, IPA, DCS, and CS obtained from
723 MEP- and MVA pathways influenced by *PtHMGR*- and *PtDXR-OEs*. **f,g,h,i, and j**, the column
724 plots reveal the effect of *PtHMGR-OE3* and *-7* and *PtDXR-OE1* and *-3* on the mentioned above
725 isoprenoids separately; NT poplars have been used as the control. Bars represent mean ± SD
726 (n = 3); Stars reveal significant differences, *P < 0.05, **P < 0.01, ***P < 0.001, ****P <

727 0.0001. k,l,m,n, and o, represent the HPLC-MS/MS chromatogram content analyses of GA3,
728 tZR, IPA, DCS, and CS, respectively affected by *PtHMGR*- and *PtDXR-OEs* compared with NT
729 poplars.

730 **Figure 5 | Phenotypic changes resulted by affected communications of MVA- and MEP**
731 **pathways amongst *PtHMGR*- and *PtDXR-OEs* in 45-day-old poplars.** **a**(I), The PtDXR
732 transgenic revealed a higher stem length than *PtHMGR-OEs* and NT poplars. **a**(II), The *PtHMGR*
733 transgenic presents an insignificantly more stem development than NT poplar. **a**(III), NT poplar
734 was used as a control; Scale bar represents 1 cm. **b**, The Box and Whisker mean comparison
735 plot of stem lengths revealed significantly higher lengths *PtDXR-OEs* than NT poplars
736 compared with *PtHMGR-OEs*. *PtHMGR* transgenics also revealed significantly higher lengths
737 than NT poplars. **c** and **d**, The Violin mean comparison plots of *ZEP* and *NCED* relative
738 expressions between *PtHMGR*-and *PtDXR-OEs* compared to NT poplars. **e**, The Box and
739 Whisker mean comparison plot of stem diameters revealed less significant differences
740 between *PtDXR-OEs* and NT poplars. Stars reveal significant differences, *P < 0.05, **P < 0.01,
741 ***P < 0.001, ****P < 0.0001.

742 **Figure 6 | Communications exist between MVA- and MEP-pathways excess of IPP and**
743 **DMAPP.** The IPP and DMAPP are considered the common precursors of the MEP- and MVA
744 pathways between cytoplasm and plastid. In addition, the putative communication generated
745 between MVA- and MEP-related genes and MVA- and MEP-derived products. MVA: mevalonic
746 acid, MEP: methylerythritol phosphate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl
747 diphosphate, AACT: acetoacetyl CoA thiolase, HMGS: 3-hydroxy-3-methylglutaryl-CoA
748 synthase, HMG-CoA: 3-hydroxy-3-methylglutary-CoA, HMGR: 3-hydroxy-3-methylglutaryl-
749 CoA reductase, MVK: mevalonate kinase, MVD: mevalonate5-diphosphate decarboxylase, IPP:
750 isopentenyl diphosphate, IDI: IPP isomerase, GPP: geranyldiphosphate, FPP:
751 famesyldiphosphate, GPS: geranyl phosphate synthase, FPS: farnesyl-diphosphate synthase,
752 GPPS: geranyl diphosphate synthase, GGPPS: geranyl geranyl diphosphate synthase, DXS: 1-
753 deoxy-D-xylulose5-phosphate synthase, DXP: 1-deoxy-D-xylulose5-phosphate, DXR: 1-deoxy-
754 D-xylulose5-phosphate reductoisomerase, HDS: 1-hydroxy-2-methyl-2-(E)-butenyl4-
755 diphosphate synthase, HDR: 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphate reductase, MCT:
756 MEP cytidylyltransferase, CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase.

757 **Supplementary figures and table**

758 **Supplementary Figure 1 | Amino acid sequences alignment of PtHMGR protein and other**
759 **known HMGR proteins.** *A. thaliana* (NP_177775.2), *G. hirsutum* (XP_016691783.1), *M.*
760 *domestica* (XP_008348952.1), *M. esculenta* (XP_021608133.1), *P. persica* (XM_020569919.1),
761 *O. sativa* (XM_015768351.2), *T. cacao* (XM_007043046.2), *Z. mays* (PWZ28886.1). The HMG-
762 CoA and NADPH binding domains are indicated in red rectangular.
763 **Supplementary Figure 2 | Construction of a phylogenetic tree based on the HMGR**
764 **sequences of various species.** Accession numbers of the HMGR obtained from Phytozome are
765 as follows: *A. thaliana* (AT1G76490 and AT2G17370) , *P. trichocarpa* (Potri.011G145000,
766 Potri.005G257000, Potri.004G208500, Potri.001G457000, Potri.009G169900 and
767 Potri.002G004000), *Gossypium raimondii* (Gorai.008G013000, Gorai.002G146000,
768 Gorai.002G014700, Gorai.005G215800, Gorai.012G138100, Gorai.005G215500,
769 Gorai.005G215600 and Gorai.005G215700), *Malus domestica* (MDP0000157996,
770 MDP0000268909, MDP0000372490, MDP0000251253 and MDP0000312032), *Manihot*
771 *esculenta* (Manes.15G114100, Manes.01G157500, Manes.03G096600, Manes.02G116900
772 and Manes.05G128600), *Oryza sativa* (LOC_Os09g31970, LOC_Os08g40180 and
773 LOC_Os02g48330), *Prunus persica* (Prupe.7G187000, Prupe.7G187500 and Prupe.8G182300),
774 *Theobroma cacao (*Thecc1EG000025, Thecc1EG007601 and Thecc1EG034814), and *Zea mays*
775 (GRMZM2G393337, GRMZM2G058095, GRMZM2G136465, GRMZM2G001645 and
776 GRMZM2G043503).
777 **Supplementary Figure 3 | Molecular identification of *PtHMGR-OEs*.** (**A**) PCR identification of
778 *PtHMGR* in *PtHMGR-OEs* and NT poplars. Lane M: 15K molecular mass marker (TransGen,
779 China); lane 1: genome DNA from WT as negative control; lanes 2–9: genome DNA from
780 *PtHMGR-OE* lines (**B**) qRT-PCR identification of the transcript levels of *PtHMGR* in *PtHMGR-*
781 *OEs* and NT poplars. Three independent experiments were performed; Stars reveal significant
782 differences, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
783 **Supplementary Figure 4 | MEP- and MVA-related genes analyses in overexpressed *PtHMG***
784 ***R*- and *PtDXR-OEs* poplars**. **a**, MVA-relate genes *AACT*, *HMGS*, *MVK*, *MVD,* and *FPS* affected b
785 y *PtHMGR* overexpressing. **b**, MVA-related genes *AACT*, *HMGS*, *HMGR*, *MVK*, *MVD,* and *FPS* a
786 ffected by *PtDXR* overexpressing. **c**, MEP-related genes *DXS*, *MCT*, *CMK*, *HDS*, *HDR*, *IDI*, *GPS, G*
787 *PPS, and DXR affected by PtHMGR* overexpressing. **d**, MEP-related genes *DXS*, *DXR*, *MCT*, *CM*
788 *K*, *HDS*, *HDR*, *IDI*, *GPS,* and *GPPS* affected by *PtDXR* overexpressing. *PtActin* was used as an in

789 ternal reference in all repeats; * P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001; Three in
790 dependent replications were performed in this experiment.
791 **Supplementary Figure 5 |** HPLC chromatograms of analyzing the contents of **(A)** β-carotene,
792 **(B)** lycopene, and **(C)** lutein in NT poplars and *PtHMGR-OEs*.
793 **Supplementary Figure 6 |** HPLC chromatograms of analyzing the contents of **(A)** β-carotene,
794 **(B)** lycopene, and **(C)** lutein in NT poplars and *PtDXR-OEs*.
795 **Supplementary Figure 7 | Chromatogram analyses of GA3 standards via HPLC-MS/MS.** The
796 chromatogram of standard GA3 at (**A**) 0.1, (**B**) 0.2, (**C**) 0.5, (**D**) 2, (**E**) 5, (**F**) 20, (**G**) 50, and (**H**)
797 200 ng/mL concentrations. (**I**) Equations for the GA3 standard curves.
798 **Supplementary Figure 8 | Chromatogram analyses of tZR standards via HPLC-MS/MS.** The
799 chromatogram of standard tZR at (**A**) 0.1, (**B**) 0.2, (**C**) 0.5, (**D**) 2, (**E**) 5, (**F**) 20, (**G**) 50, and (**H**)
800 200 ng/mL concentrations. (**I**) Equations for the tZR standard curves.
801 **Supplementary Figure 9 | Chromatogram analyses of IPA standards via HPLC-MS/MS.** The
802 chromatogram of standard IPA at (**A**) 0.2, (**B**) 0.5, (**C**) 2, (**D**) 5, (**E**) 20, (**F**) 50, and (**G**) 200 ng/mL
803 concentrations. (**H**) Equations for the IPA standard curves.
804 **Supplementary Figure 10 | Chromatogram analyses of DCS standards via HPLC-MS/MS.** The
805 chromatogram of standard DCS at (**A**) 0.5, (**B**) 2, (**C**) 10, (**D**) 20, and (**E**) 50 ng/mL concentrations.
806 (**F**) Equations for the DCS standard curves.
807 **Supplementary Figure 11 | Chromatogram analyses of CS standards via HPLC-MS/MS.** The
808 chromatogram of standard CS at (**A**) 0.5, (**B**) 5, (**C**) 10, (**D**) 20, and (**E**) 50 ng/mL concentrations.
809 (**F**) Equations for the CS standard curves.
810 **Supplementary Table 1 |** Primers were used in this study.
811 **Supplementary Table 2 |** Table of data analyses used in phenotypic changes evaluation. **a**,
812 Stem diameter data analyses. **b**, Stem length data analyses**.**

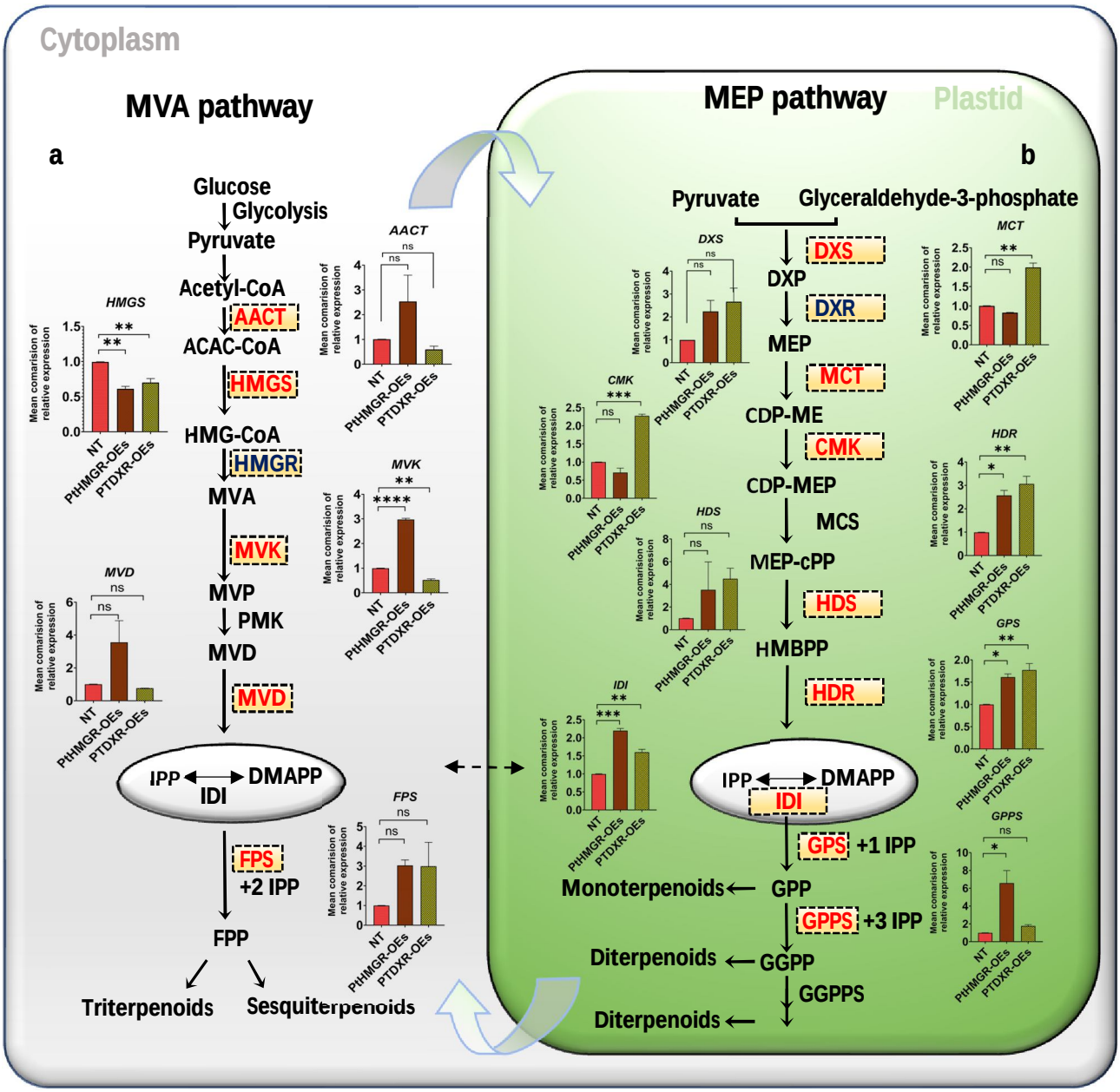


Figure 1 | MVA- and MEP-related genes analyses in overexpressed *PtHMGR*- and *PtDXR-OEs* poplars. a, Mean comparison of relative expression of MVA-relate genes *AACT*, *HMGS*, *MVK*, *MVD,* and *FPS* (Indicated in red) affected by *PtHMGR* overexpressing. **b**, Mean comparison of relative expression of MEP-related genes *DXS*, *MCT*, *CMK*, *HDS*, *HDR* , *IDI*, *GPS,* and *GPPS* (Indicated in red) affected by *PtHMGR* overexpressing; *HMGR* and *DXR*, which were overexpressed respectively by *DXR*- and *HMGR-OEs,* were presented in Supplementary Figure 4. *PtActin* was used as an internal reference in all repeats; "ns" means not significant, * P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001; Three independent replications were performed in this experiment.

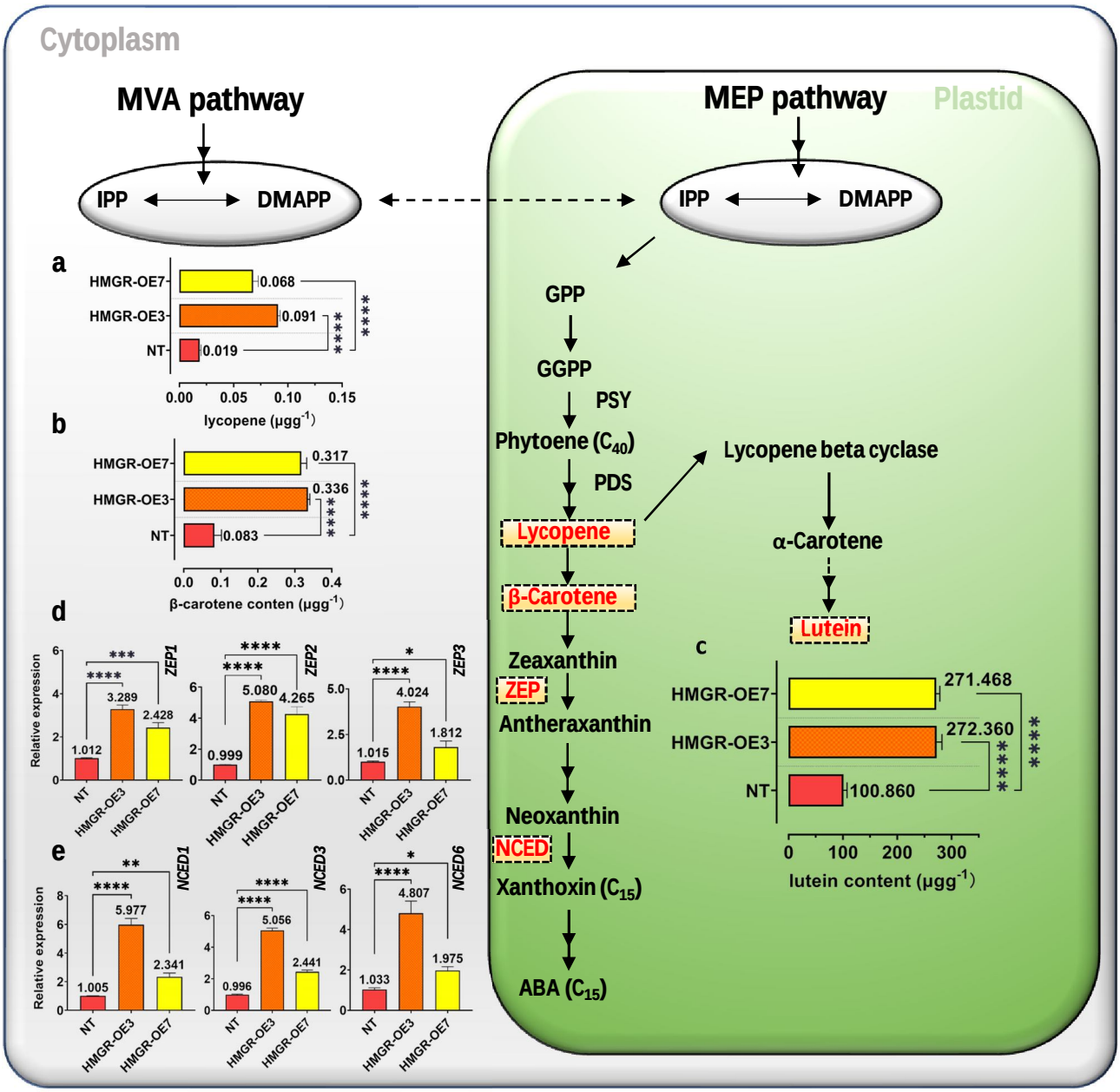


Figure 2 | HPLC-MS/MS content analyses of lycopene, β-carotene, lutein, and real-time PCR of *ZEP* and *NCED* genes family. HPLC-MS/MS content analyses have been performed to show the effect of *PtHMGR-OEs* on **a**, lycopene **b**, β-carotene, and **c**, lutein. Relative expressions have been analyzed affected by *PtHMGR-OEs* compared with NT poplars of **d**, *ZEP*, and **e**, *NCED* genes family. Bars represent mean ± 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.

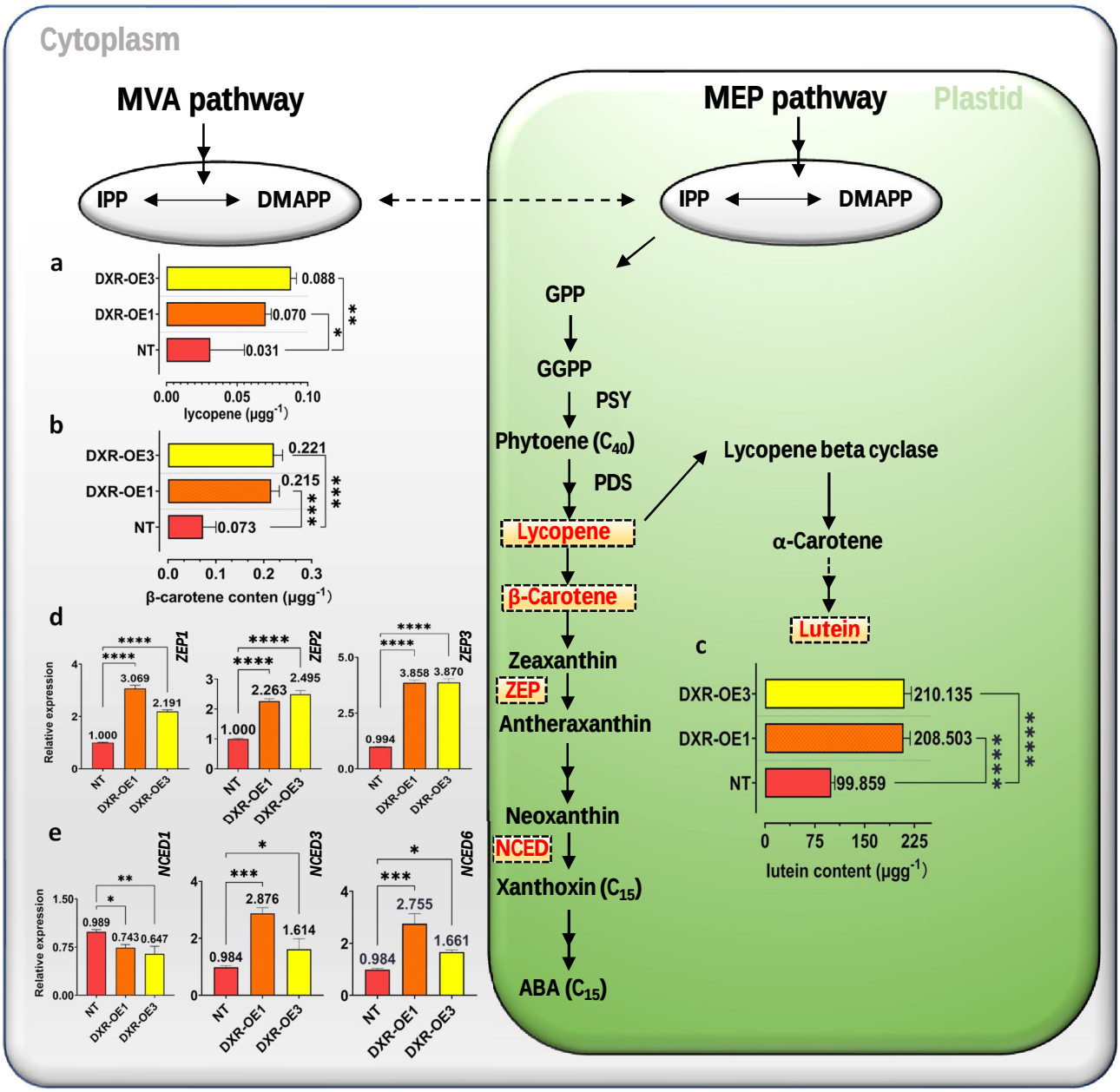
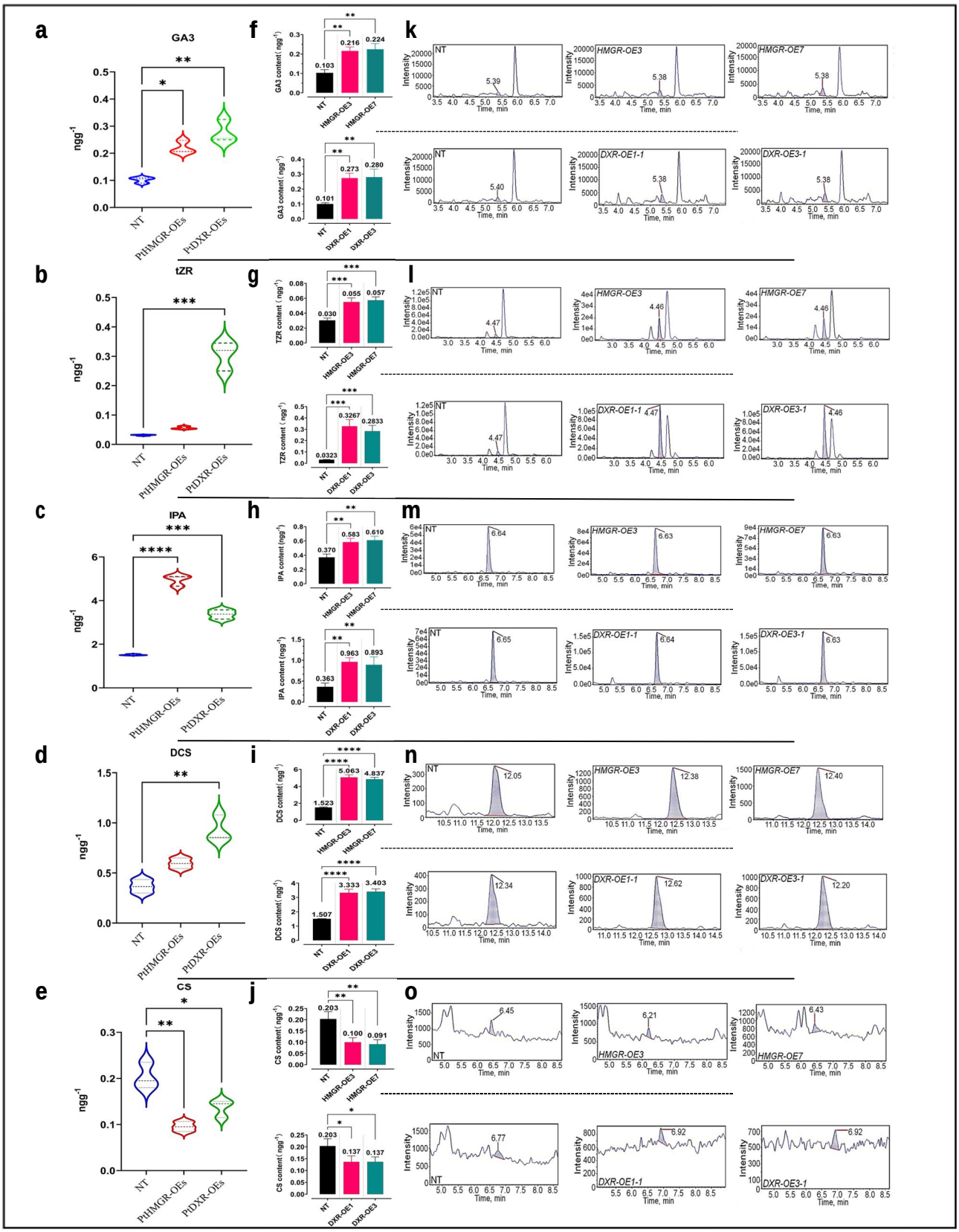


Figure 3 | HPLC-MS/MS content analyses of lycopene, β-carotene, lutein, and real-time PCR of *ZEP* and *NCED* genes family. HPLC-MS/MS content analyses have been performed to show the effect of *PtDXR-OEs* on **a**, lycopene **b**, β-carotene, and **c**, lutein. Relative expressions have been analyzed affected by *PtDXR-OEs* compared with NT poplars of **d**, *ZEP*, and **e**, *NCED* genes family. Bars represent mean ± 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.



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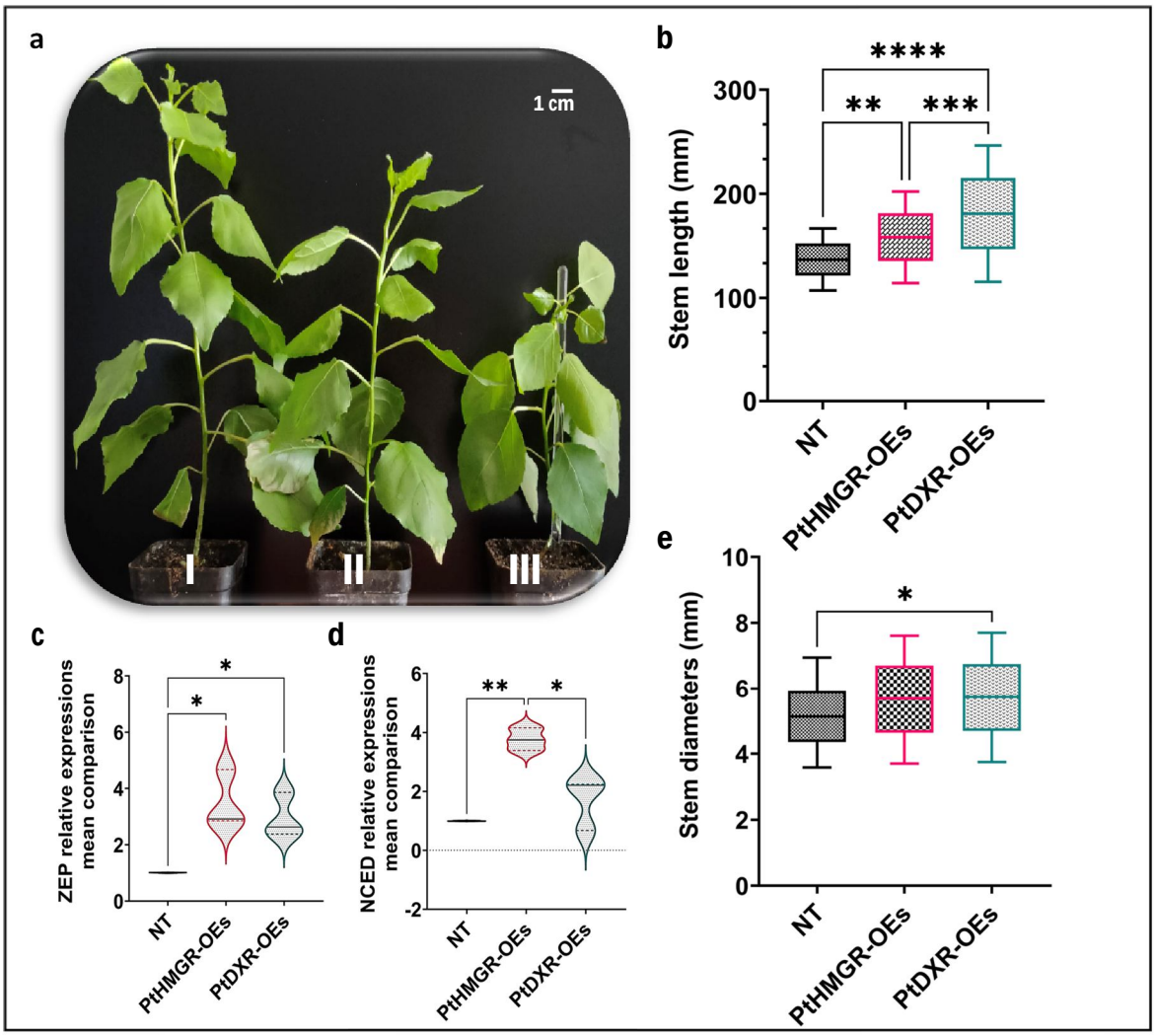

Figure 5 | Phenotypic changes resulted by affected communications of MVA- and MEP pathways amongst *PtHMGR*- and *PtDXR-OEs* in 45-day-old poplars. a(I), The PtDXR transgenic revealed a higher stem length than *PtHMGR-OEs* and NT poplars. **a**(II), The *PtHMGR* transgenic presents an insignificantly more stem development than NT poplar. **a**(III), NT poplar was used as a control; Scale bar represents 1 cm. **b**, The Box and Whisker mean comparison plot 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. **c** and **d**, The Violin mean comparison plots of *ZEP* and *NCED* relative expressions between *PtHMGR*-and *PtDXR-OEs* compared to NT poplars. **e**, The Box and Whisker mean comparison plot 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.

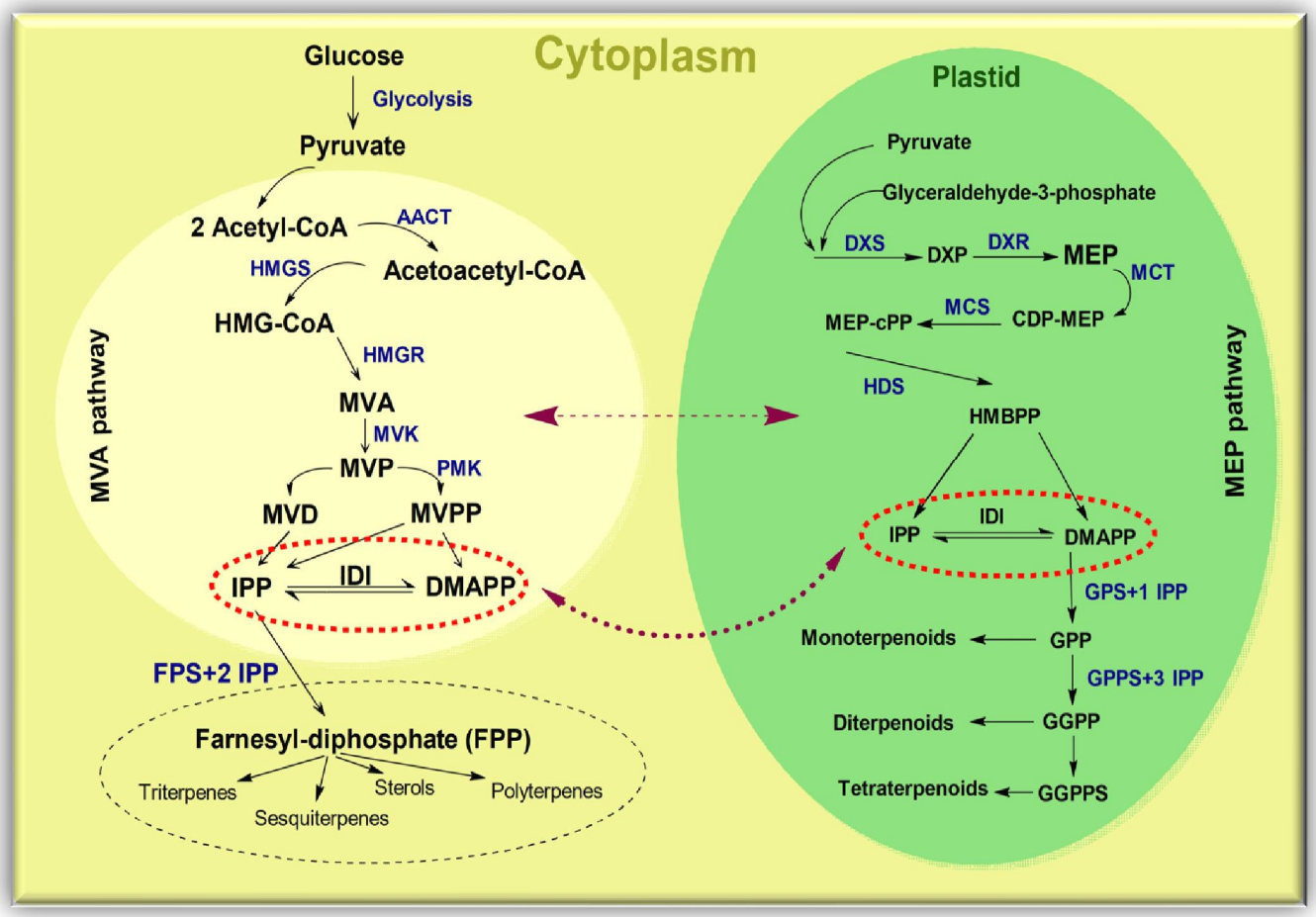

Figure 6 | Communications exist between MVA- and MEP-pathways excess of IPP and DMAPP. The IPP and DMAPP are considered the common precursors of the MEP- and MVA pathways between cytoplasm and plastid. In addition, the putative communication generated between MVA- and MEP-related genes and MVA- and MEP-derived products. MVA: mevalonic acid, MEP: methylerythritol phosphate, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, AACT: acetoacetyl CoA thiolase, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMG-CoA: 3-hydroxy-3-methylglutary-CoA, 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 cytidylyltransferase, CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase.