Metabolic engineering of *Barbarea vulgaris* identifies β-amyrin synthase LUP5 as a determinant of saponin-mediated insect resistance

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Summary

• Plant–insect coevolution drives the diversification of specialized metabolites, yet the genetic basis of ecotypic variation in defensive chemistry remains poorly understood. The wild crucifer *Barbarea vulgaris* comprises two ecotypes—an insect-resistant G-type and a susceptible P-type—characterized by distinct triterpenoid saponin profiles.

• To investigate the causal role of saponin composition in insect resistance, we developed a stable transformation system for *B. vulgaris*. We expressed the G-type β-amyrin synthase gene (*LUP5*) in both ecotypes and silenced the cytochrome P450 gene *CYP72A552* in the G-type. We combined metabolic profiling with insect feeding assays to assess functional outcomes.

• Ectopic expression of G-type *LUP5* in the susceptible P-type conferred up to 95% reduction in *Plutella xylostella* feeding, accompanied by increased accumulation of three hederagenin-derived monodesmosidic saponins. In contrast, silencing of *CYP72A552* reduced hederagenin derived saponin levels but did not affect herbivory, suggesting threshold-dependent resistance.

• We provide direct *in planta* evidence that *LUP5* is a key determinant of ecotypic insect resistance in *B. vulgaris*. Our findings establish a functional link between oxidosqualene cyclase activity, saponin structural diversification, and ecological defense, highlighting the roles of both sapogenin backbone and glycosylation pattern in saponin-mediated herbivore deterrence.

Keywords: triterpenoid saponins, metabolic engineering, β-amyrin synthase, insect resistance, *Barbarea vulgaris,* chemical ecology

36 **Introduction**

37 Plants have evolved a wide array of specialized metabolites to deter insect
38 herbivores (Fürstenberg-Hägg, Zagrobelny and Bak, 2013; Yactayo-Chang et al., 2020;
39 Beran and Petschenka, 2022). Among these, triterpenoid saponins, which consist of a
40 hydrophobic sapogenin backbone and one or more hydrophilic sugar moieties, represent
41 a structurally diverse and ecologically important class of defensive compounds. Due to
42 their structural similarity to sterols and steroid hormones, triterpenoid saponins have
43 been hypothesized to interact with eukaryotic cell membranes. In addition, their inherent
44 bitterness may deter herbivores, contributing to their role as effective defense agents
45 (Augustin et al., 2011). Thousands of triterpenoid saponins have been reported to date
46 (Xu, Fazio and Matsuda, 2004; Augustin et al., 2011; Moses, Papadopoulou and Osbourn,
47 2014; Huang et al., 2020). Importantly, specific saponin structures have been implicated
48 in insect resistance, highlighting the functional significance of their chemical diversity
49 (Stevenson, Nyirenda and Veitch, 2010; Cui et al., 2019; Hussain et al., 2019; Liu et al.,
50 2019; Dolma et al., 2021).

51 The structure–activity relationships underlying saponin bioactivity are critical to their
52 defensive function. For example, hederagenin 3-*O*-monoglucoside has higher toxicity and
53 antifeedant activity than oleanolic acid 3-*O*-monoglucoside (which lacks C-23
54 hydroxylation), and gypsogenic acid 3-*O*-monoglucoside (which is further oxidized at C-23
55 to the ketone) (Liu et al., 2019). Additionally, monodesmosidic hederagenin and oleanolic
56 acid exhibited higher toxicity and antifeedant activity than their bidesmosidic
57 counterparts (Tian et al., 2021). These observations suggest that even subtle
58 modifications to the sapogenin backbone, glycosylation pattern, or sugar linkage can
59 significantly alter saponin bioactivity. This structural diversity makes saponins a valuable
60 system for studying molecular evolutionary mechanisms of specialized metabolism,
61 particularly the structure–activity relationships underlying their ecological functions in
62 insect defense. Despite growing knowledge of these structure–activity relationships, the
63 genetic and biochemical mechanisms underlying saponin diversification remain poorly

64 understood in most species. One particularly promising ecological model to explore this
65 question is the wild crucifer *Barbarea vulgaris*, which naturally segregates in two ecotypes
66 or chemotypes that alter in both saponin profiles and insect resistance.

67 *B. vulgaris* has emerged as a powerful ecological model for investigating how
68 specialized metabolites contribute to herbivore resistance (Kuzina et al., 2011; Hauser,
69 Toneatto and Nielsen, 2012; Byrne et al., 2017). The species comprises two ecotypes: the
70 glabrous insect-resistant G-type, and the pubescent insect-susceptible P-type (Agerbirk,
71 Olsen and Nielsen, 2001). This intraspecific divergence likely arose from isolation in
72 different Ice Age refugia (Hauser, Toneatto and Nielsen, 2012) and is associated with
73 marked differences in saponin composition (Kuzina et al., 2009; Khakimov et al., 2012;
74 Khakimov et al., 2016). Gene duplications events have further contributed to the chemical
75 divergence and insect resistance (Khakimov et al., 2015; Liu et al., 2019). Insect resistance
76 in G-type *B. vulgaris* correlates with four oleanane-type defensive saponins, including
77 oleanolic acid cellobioside, hederagenin cellobioside, gypsogenin cellobioside, and 4-
78 epihederagenin cellobioside (Agerbirk et al., 2003; Kuzina et al., 2011). Among these,
79 hederagenin cellobioside is particularly toxic and confers G-type insect resistance
80 (Agerbirk et al., 2003; Nielsen et al., 2010; Kuzina et al., 2011).

81 The biosynthetic pathway leading to hederagenin cellobioside in G-type *B. vulgaris*
82 has been partially elucidated. The oxidosqualene cyclase *LUP5* is highly expressed in the
83 G-type and primarily produces β-amyrin (Khakimov et al., 2015). Subsequent enzymatic
84 steps involve CYP716A80, CYP72A552, and UGT73C11, which sequentially convert β-
85 amyrin into oleanolic acid, hederagenin, and hederagenin 3-*O*-glucoside (Augustin et al.,
86 2012; Khakimov et al., 2015; Liu et al., 2019). The enzyme responsible for the final step in
87 forming hederagenin cellobioside remains unidentified (Erthmann, Agerbirk and Bak,
88 2018). In contrast, *LUP5* is expressed at very low levels in *B. vulgaris* P-type and primarily
89 produces α-amyrin due to two amino acid substitutions as compared to the *LUP5* in the
90 G-type (Günther et al., 2021). Despite 98% sequence identity at the amino acid level, the
91 G- and P-type LUP5 enzymes differ markedly in both their gene expression and product

92 profiles. The P-type plants preferentially express *LUP2,* an oxidosqualene cyclase over
93 80% identical at the amino acid level to *LUP5*, but it produces predominantly lupeol (98%)
94 and only 2% β-amyrin-derived saponins (Khakimov et al., 2015). Notably, the genes
95 encoding for the enzymes responsible for the biosynthesis of hederagenin 3-*O*-glucoside
96 (CYP716A80, CYP72A552, and UGT73C11) are present in both P-type and G-type and
97 exhibit similar substrate and product specificity (Augustin et al., 2012; Khakimov et al.,
98 2015; Liu et al., 2019). However, the cyclases are differentially regulated suggesting that
99 upstream regulation and oxidosqualene cyclase product specificity identity may be key
100 drivers of saponin structural and insect resistance difference. Additionally, CYP72A552,
101 which catalyzes the C23 hydroxylation of oleanolic acid to hederagenin, the precursor of
102 the more toxic hederagenin-type saponins, may also be pivotal to defense against insects,
103 but its precise role in *B. vulgaris* defense remains to be experimentally validated.

104 Despite these prior insights, a direct causal link between saponin composition and
105 insect resistance has not yet been established *in planta*. Here, we leverage the natural
106 variation in *B. vulgaris* to develop a model system that connects specialized metabolism
107 with ecological function. We provide functional evidence that engineering saponin
108 biosynthesis through *LUP5* manipulation can substantially enhance insect resistance in
109 this wild crucifer and explains the molecular basis of the difference in insect resistance
110 between the G- and P-type *B. vulgaris.*

111 **Materials and methods**

112 **Plant and insect materials**

113 Seeds of *Barbarea vulgaris* G-type (glabrous, accession B44) and P-type (pubescent,
114 accession B4) were provided by Associate professor Niels Agerbirk *(Agerbirk et al., 2021).*
115 *Brassica napus* plants were grown in a greenhouse at 19°C under a 16:8 h light-dark cycle.
116 *Plutella xylostella* (diamondback moth) was reared on *B. napus* at 20°C under the same
117 light conditions. Third instar *P. xylostella* larvae were used for the insect feeding assay.

118 **Plasmids construction**

119 For stable transformation, the pJCV51 vector was employed to introduce the G-type
120 *LUP5* gene, while pK7FWG2 was utilized for *CYP72A552* silencing. The *LUP5* gene, its
121 native promoter (1988 bp), and the *CYP72A552* gene were PCR-amplified from *B. vulgaris*
122 G-type cDNA and genomic DNA (Table S1, Table S2). G-type *LUP5* expression was driven
123 either by the 35S promoter or its native promoter, while *CYP72A552* was driven by the
124 35S promoter.

125 The pJCV51-p35S::LUP5 and pK7FWG2-CYP72A552 RNAi plasmids were assembled
126 using Gateway technology (Gateway™ BP Clonase™ II Enzyme mix, Thermo Fisher
127 Scientific, 11789020; Gateway™ LR Clonase™ II Enzyme mix, Thermo Fisher Scientific,
128 11791020) (Table S1). pDONR207 was used as the entry clone vector for both constructs
129 (Thermo Fisher Scientific, 117207-021). Subsequently, the pJCV51-pLUP5::LUP5 plasmid
130 was generated by replacing the 35S promoter in pJCV51-p35S::LUP5 with the G-type *LUP5*
131 promoter using the SalI sites. Finally, the plasmids were introduced into *Agrobacterium*
132 *tumefaciens* strain AGL1 via electroporation (Hayta et al., 2021).

133 **Stable transformation in wild crucifer *B. vulgaris***

134 Seed sterilization was achieved by immersing seeds in 70% ethanol for 30 s, followed
135 by a 12.5 min treatment in 3% sodium hypochlorite (Thermo Fisher Scientific) with 0.1%
136 Tween20 (Bio-RAD, 1706531) and thorough rinsing with sterile water. Seeds were sown
137 on germination medium in Magenta boxes and incubated in darkness for 6–8 days (Table
138 S3) .

139 Hypocotyl explants (~3 mm) from 6–10-day-old seedlings were incubated for 15 min
140 with *A. tumefaciens* strains (OD_{600} = 0.5 in 10 mM $MgCl_2$, 100 µM acetosyringone) with
141 gentle agitation, and then co-cultivated in darkness for three days (Table S3). The pJCV51-
142 pLUP5::LUP5 and pJCV51-35S::LUP5 constructs were transformed into both *B. vulgaris*
143 ecotypes, while pK7FWG2-CYP72A552 RNAi was only introduced into the G-type.

144 After rinsing in Milli-Q water containing 1 µL/mL timentin, the explants were
145 transferred sequentially to callus induction (one week), shoot induction (two rounds, two

146 weeks each), and root induction media (two rounds, two weeks each) at 25°C under a
147 16:8 h light–dark cycle. Throughout these stages, the explants were maintained in a
148 climate chamber at 25°C under a 16:8 h light-dark cycle. Kanamycin (50 ng/mL) was used
149 for selecting transformed explants, whereas wild-type explants were cultured without
150 kanamycin supplementation in the medium. Regenerated plants were confirmed by
151 DsRed fluorescence (for *LUP5* expression constructs) or NPTII (neomycin
152 phosphotransferase II) ELISA Kit (Agdia, PSP 73000/0288) for CYP72A552 RNAi.
153 Subsequently, the confirmed plants were transferred to soil and grown in a greenhouse
154 at 19°C under a 16:8 h light–dark cycle.

155 **Choice insect feeding assay**

156 Insect resistance was evaluated using at least three individual plants (biological
157 replicates) per transgenic line. For each transgenic plant, five transgenic and five wild-
158 type leaf discs (1.57 cm^2 each) were alternately arranged in a PETRI DISH (9.4 x 1.6 cm)
159 (Greiner BIO-ONE, 633180) with filter paper moistened with 2 mL of water. After a 3–5
160 hour starvation period, ten third-instar *P. xylostella* larvae were placed at the center of
161 the plate, following a modified protocol from Liu et al (Liu et al., 2019). Leaf consumption
162 was quantified when half of the wildtype leaves were consumed using ImageJ (approx 7.5
163 h).

164 **Preparation of plant extracts and metabolite analysis**

165 The extraction of saponins and sapogenins was adapted from Khakimov *et al*. (2016).
166 *B. vulgaris* leaf samples were ground with liquid nitrogen using a mortar and pestle. For
167 saponin detection, 100 mg of leaf powder was weighed into 1.5 mL Eppendorf tubes and
168 extracted with 300 µL of 85% methanol (v/v) via sonication for 30 minutes, followed by
169 centrifugation at 16,000 g for 10 minutes. Supernatants (200 µL) were filtered through a
170 0.22-µm filter before LC-qTOF-ESI-MS/MS analysis.

171 To detect total and free sapogenins, 200 mg of leaf powder was weighed into 2 mL
172 Eppendorf tubes and extracted with 600 µL of 85% methanol (v/v) at 100°C while mixing

173 at 1,400 rpm in a thermomixer (Eppendorf, Denmark). The samples were cooled on ice
174 and centrifuged at 16,000 × *g* for 3 min. After filtration through a 0.22-µm filter, 100 µL
175 of supernatant was collected for free sapogenin detection via LC-qTOF-APCI-MS/MS. An
176 additional 300 µL of supernatant was collected and dried under nitrogen flow. To cleave
177 off sugar residues, samples were treated with 500 µL of 2 M hydrochloric acid at 100°C
178 while mixing at 1400 rpm for 1.5 hours in a thermomixer (Eppendorf, Denmark). After
179 cooling on ice, a double volume of ethyl acetate was added to the acid-water mixture,
180 followed by vortexing and centrifugation at 3,500 × *g* for 5 minutes. The samples were
181 extracted three times with ethyl acetate to collect sapogenins. To remove residual acid,
182 an equal volume of Milli-Q water was added, the samples was subsequently vortexed and
183 centrifugated at 3,500 × *g* for 5 minutes. This washing step was repeated three times. The
184 final extracts were dried under nitrogen flow, re-solubilized in 300 µL of 100% methanol,
185 and filtered through a 0.22-µm filter before total sapogenin detection via LC-qTOF-APCI-
186 MS/MS.

187 LC-qTOF-ESI-MS/MS was performed according to Liu *et al*. (2019). LC-qTOF-APCI-
188 MS/MS was performed as described by Patel *et al*. (2020). Untargeted metabolite data
189 were analyzed using XCMS (Tautenhahn et al., 2012), and targeted saponin and sapogenin
190 analysis were performed according to Khakimov *et al*. (2016). Hederagenin cellobioside
191 and oleanolic acid cellobioside standards were used for saponin identification. Lupeol, β-
192 amyrin, oleanolic acid, and hederagenin standards were employed for sapogenin
193 identification.

194 **Gene expression analysis**

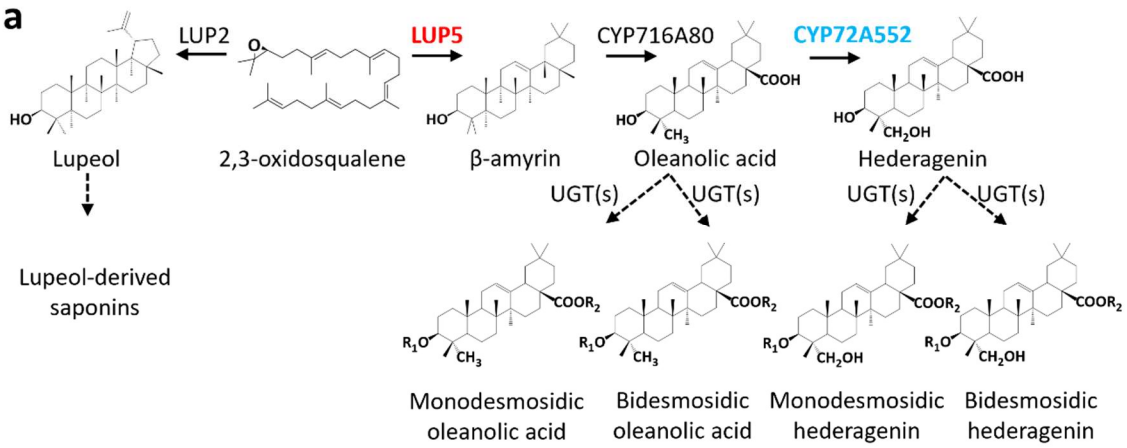
195 Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich,
196 STRN50-1KT) and then synthesized to cDNA by iScript™ cDNA Synthesis Kit (Bio-Rad,
197 1708891). Relative expression of *LUP5* was quantified via qPCR using kapa SYBR® fast kit
198 (Merck-Sigma, KK4607) (Khakimov et al., 2015). *Tubulin* (GenBank accession no.
199 EU555191) was used as reference gene, and *LUP5* expression levels were normalized to

200 the wild-type P-type control. Primer sequences are provided in Table S1 (Khakimov et al.,
201 2015).

202 **Results**

203 **Establishment of a transformation and regeneration system in wild crucifer *B. vulgaris***

204 To enable functional studies of insect resistance in *B. vulgaris* as an ecological model
205 system, we developed a stable transformation and regeneration method for both the G-
206 type and P-type ecotypes (Fig.1a, Fig. S1, Table S3). To metabolically engineer the saponin
207 compositions, we introduced the G-type *LUP5* gene into both ecotypes and attempted to
208 silence *CYP72A552* in the G-type. *LUP5* expression was driven either by the constitutive
209 35S promoter or the native *LUP5* promoter. Transformation efficiency, calculated as the
210 percentage of positive transgenic plants to all obtained plants from tissue culture,
211 reached up to 94% across experiments (Fig. 1b). The native *LUP5* promoter exhibited
212 approximately two-fold higher transformation efficiency than the 35S promoter in both
213 the G- and P-type, indicating a selection pressure against overexpression *LUP5* using the
214 35S-promoter (Fig. 1b).



b

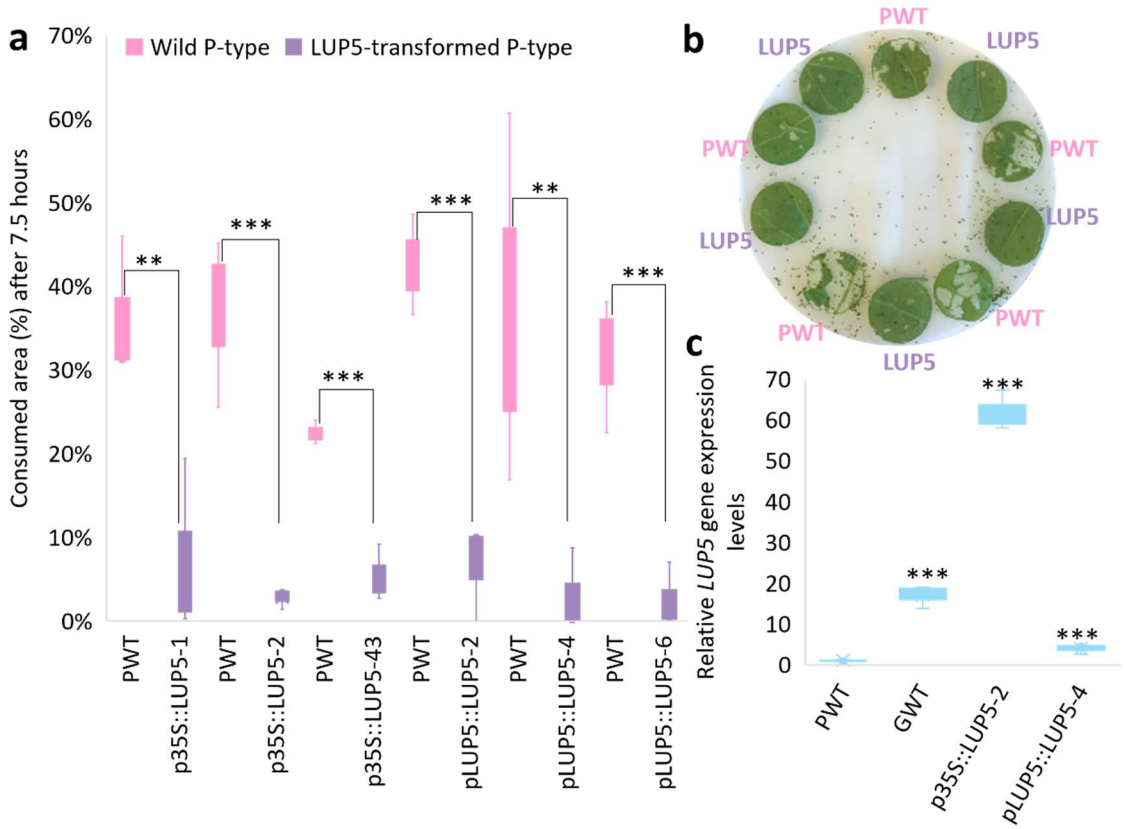
Construct for transformation	Ecotype	Total numbers of plants	Positive/total
pJCV51-pLUP5::LUP5	G-Type	31	93.54%
	P-Type	35	60.00%
pJCV51-p35S::LUP5	G-Type	52	40.4%
	P-Type	66	25.8%
pK7FWG2-CYP72A552 RNAi	G-Type	46	28.26%

Fig. 1 Triterpenoid saponin biosynthesis pathways and stable transformation efficiency in *B. vulgaris*. (a) Biosynthetic pathways for lupeol and β–amyrin derived triterpenoid saponins in *B. vulgaris*. Monodesmosidic saponin, a single sugar chain attached at either C-3 (R1) or C-28 (R2); bidesmosidic saponin, sugar chains attached at both C-3 (R1) and C-28 (R2). (b) Transformation efficiency in *B. vulgaris*.

Stably expression of G-type *LUP5* in the P-type confers insect resistance

To evaluate the impact of G-type *LUP5* expression on insect resistance, a choice insect feeding assay was conducted by presenting alternating wildtype and transgenic *B. vulgaris* leaf discs to diamondback moth larvae. Leaf area consumed was measured when approximately half of the total leaf material of the wild type had been eaten (approx. 7.5 hr). *LUP5*-transformed P-type plants exhibited a significant reduction in larval consumption, ranging from 76- 95% (Fig. 2a,b). QPCR on the best performing 35S and LUP5 promoter lines, confirmed that the G-type *LUP5* gene was transformed and expressed in the P-type both under the 35S promoter or the native G-type *LUP5* promoter (Fig. 2c). Interestingly, the 35S promoter expressed the *LUP5* gene at approximately

231 sixteen-fold higher levels than that in the best pLUP5::LUP5 transformed P-type and the
232 wildtype P- and G-type (Fig. 2c).



233

234 Fig. 2 *B. vulgaris* P-type plants expressing the G-type *LUP5* gene leads to reduced feeding
235 by diamond back moth larvae. (a) Consumption area (%) after 7.5 h (choice test). (b)
236 Example of the insect choice feeding assay, and the resulting leaf condition after 7.5 hr
237 insect exposure. (c) Relative *LUP5* gene expression levels. PWT, wildtype P-type *B. vulgaris*;
238 GWT, wildtype G-type *B. vulgaris*; p35S::LUP5, p35S::LUP5 transformed P-type *B. vulgaris*;
239 pLUP5::LUP5, pLUP5::LUP5 transformed P-type *B. vulgaris*, LUP5, P-type *B. vulgaris*
240 expressing the G-type *LUP5* gene. Statistical significance is indicated by ** ($p < 0.05$) and
241 *** ($p < 0.005$).

242

243

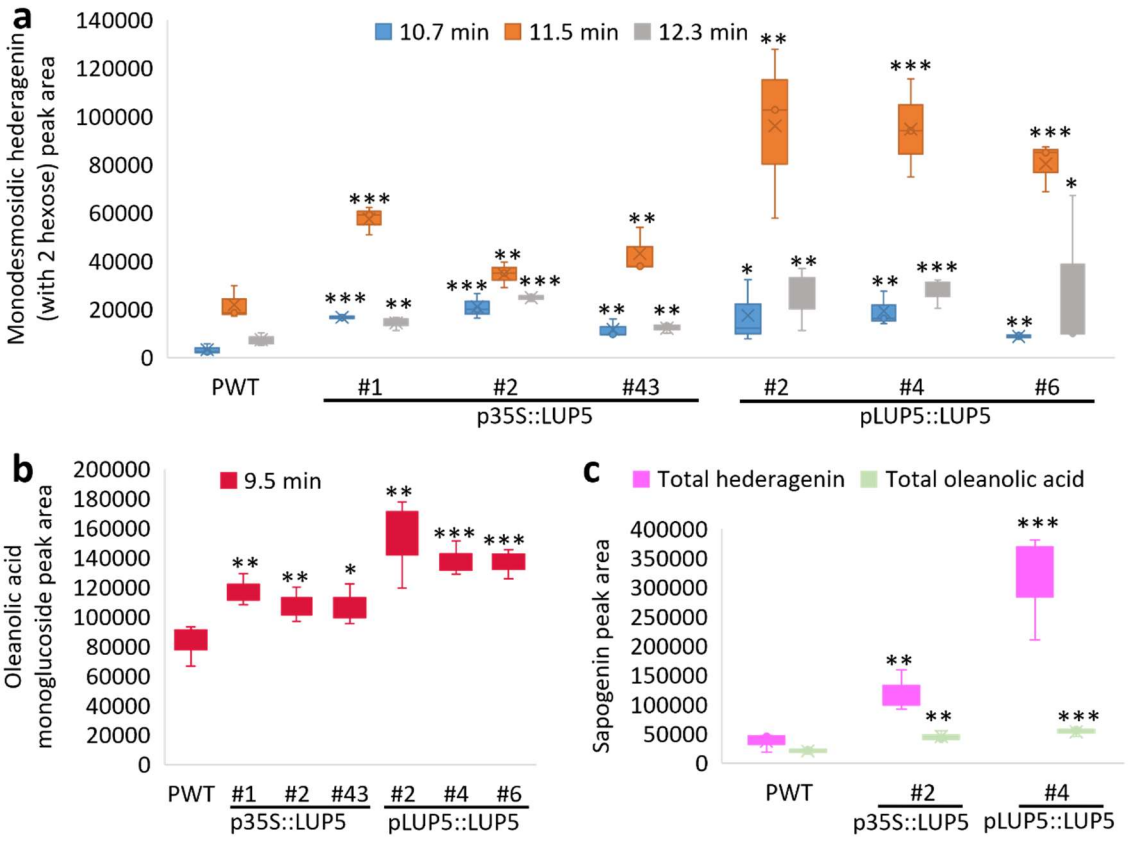
244 **Expression of G-type *LUP5* gene induced β-amyrin-derived saponins in P-type *B.***
245 ***vulgaris*.**

246 Based on the highest reduction of leaf area consumed, three transgenic lines were
247 selected for each promoter type (35S and native *LUP5*) and subjected to untargeted and
248 targeted LCMS analyses to assess metabolite changes. The untargeted metabolite
249 analysis revealed approximately 9,500 distinct m/z features—each defined as a unique
250 m/z and retention time (RT) pair. The top 100 significantly changed features (P<0.5) were
251 identified by comparing *LUP5*-expressing P-type with wild-type. However, extraction of
252 the m/z features using DataAnalysis software revealed that the majority lacked clear MS^2
253 fragmentation patterns, thus structural identification could not be deduced. This
254 outcome suggests that many of the significantly altered features may represent in-source
255 fragments, background ions, or uncharacterized metabolites for which fragmentation
256 data were inconclusive.

257 Consequently, a targeted saponin analysis was conducted using DataAnalysis. *B.*
258 *vulgaris* saponins were detected by extracted ion chromatograms of our previously
259 tentatively identified *B. vulgaris* aglycones and their structures were inferred based on
260 fragmentation patterns. Based on 455 m/z, 469 m/z and 471 m/z, the characteristic
261 fragmentation patterns of oleanolic acid, gypsogenin and hederagenin were tentatively
262 identified, respectively. This targeted approach identified ten distinct saponins in P-type
263 lines and thirty in G-type lines, which all included in the untargeted analysis data (Table
264 S4, Table S5). All detected saponins were classified as monodesmosidic, based on their
265 fragmentation patterns. Saponin abundance was subsequently quantified by calculating
266 peak areas from extracted ion chromatograms (Table S4, Table S5).

267 The LCMS analysis showed that three hederagenin-derived monodesmosidic
268 saponins (each with two hexoses) at retention times 10.7, 11.5, 12.3, and one oleanolic
269 acid monoglucoside (with one hexose) at retention time 9.5 min, accumulated in
270 significantly higher amount in *LUP5*-transformed P-type as compared to wildtype P-type
271 (Fig. 3a,b). The most abundant of the three hederagenin-derived saponin eluted at 11.5

272 min and was increased ~four-fold in the P-type expressing *LUP5* under the native *LUP5*
273 promoter compared to the wildtype and was ~two-fold more abundant than in P-type
274 plants transformed with *LUP5* under the 35S promoter (Fig. 3a). Hederagenin cellobioside
275 (eluted at 12.3 min) was also increased ~four-fold but remained at lower levels in
276 pLUP5::LUP5 transformed P-type (Fig. 3a). These three hederagenin-derived saponins
277 were also present in wild G-type plants, with hederagenin cellobioside (eluted at 12.3 min)
278 being the most abundant hederagenin-derived saponin (Table S5, Table S8). The
279 increased oleanolic acid monoglucoside (eluted at 9.5 min) was not detected in G-type
280 (Table S5). The other detected saponins remained unchanged (Table S6).



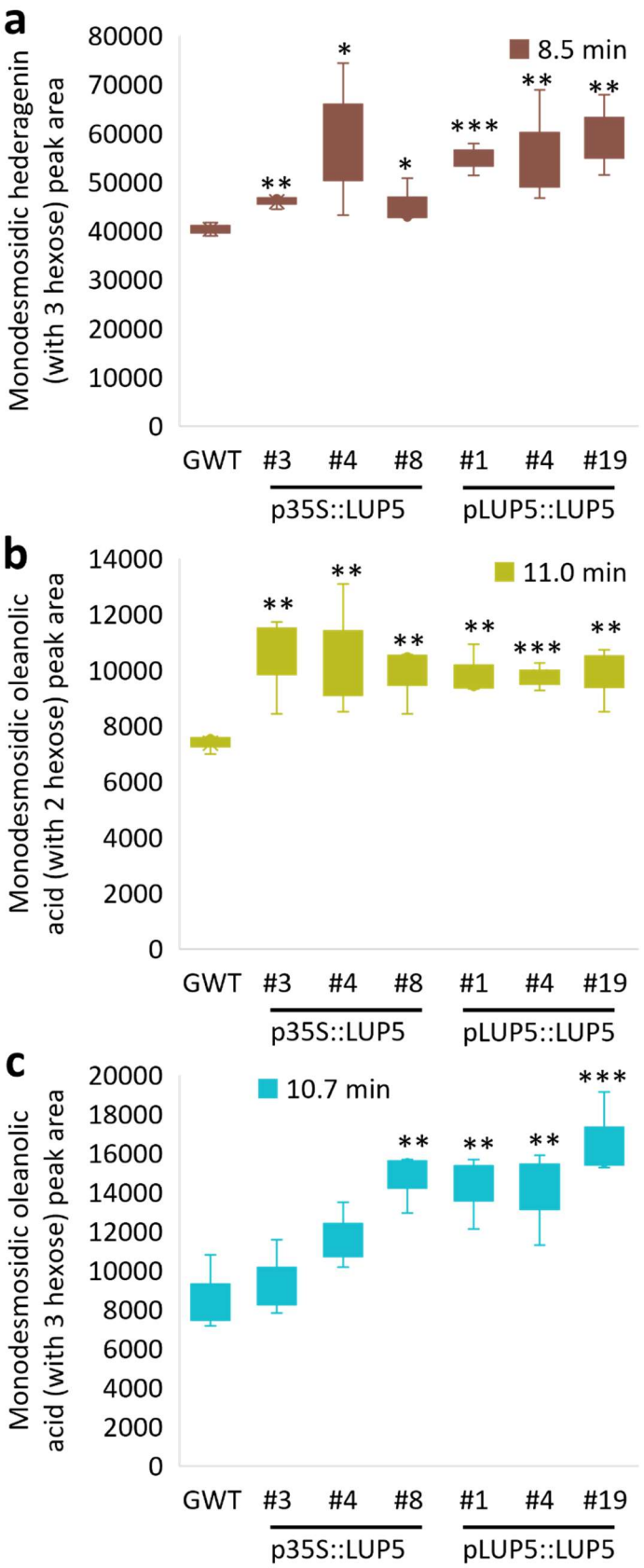
281

282 Fig. 3 Expression of the G-type *LUP5* gene increased glycosylated oleanolic acid and
283 hederagenin levels in the transgenic P-type *B. vulgaris*. (a) Monodesmosidic hederagenin
284 (with 2 hexoses) peak area (EIC: m/z = 841.4591 ± 0.2). (b) Oleanolic acid monoglucoside
285 peak area (EIC: m/z = 617.4059 ± 0.2). (c) Peak area of sapogenins, including total

286 hederagenin (EIC: m/z = 437.3425 ± 0.2) and total oleanolic acid (EIC: m/z = 439.3593±
287 0.2). PWT, wildtype P-type *B. vulgaris*; p35S::LUP5, p35S::LUP5 transformed P-type *B.*
288 *vulgaris*; pLUP5::LUP5, pLUP5::LUP5 transformed P-type *B. vulgaris*. Statistical
289 significance is indicated by *(p<0.5), ** (p < 0.05) and *** (p < 0.005).

290 To evaluate the effect of G-type *LUP5* expression on P-type *B. vulgaris* saponin
291 aglycone biosynthesis and accumulation, levels of free and total sapogenins were
292 analyzed. Free sapogenins were measured from untreated samples, representing
293 aglycones that occur naturally in a non-glycosylated form. Total sapogenins were defined
294 as the sum of free and sugar-bound sapogenins, the latter released through hydrolysis of
295 glycosylated saponins. A transgenic line (with three vegetative clones) from both
296 p35S::*LUP5* and pLUP5::*LUP5*-transformed P-type *B. vulgaris* was selected based on
297 reduced leaf area consumption and increased accumulation of four saponins (Fig. 2a, Fig.
298 3a,b). This analysis revealed a significant increase in total hederagenin and oleanolic acid
299 levels in *LUP5*-transformed P-type plants (Fig. 3c), while total lupeol, β-amyrin, and free
300 sapogenins remained undetectable (Table S7). Higher accumulation of total hederagenin
301 was observed in plants expressing *LUP5* under the native promoter compared to those
302 under the 35S promoter (Fig. 3c).

303 Introduction of G-type *LUP5* in G-type increased a monodesmosidic hederagenin
304 (with three hexoses) and oleanolic acid monoglucoside (with two hexoses) in both
305 p35S::LUP5 and pLUP5::LUP5 transformed G-type plants, retention times 8.5, and 11.0
306 min, respectively (Fig. 4a-c). Monodesmosidic oleanolic acid (with 3 hexose, retention
307 time at 10.7 min) increased in all pLUP5::LUP5 transformed G-type lines (Fig. 4c). Twenty-
308 six other saponins showed no changes (Table S8). The three slightly increased saponins
309 remained at a low concentration compared to other saponins (Fig. 4 a-c, Table S8).


a
Monodesmosidic hederagenin (with 3 hexose) peak area
8.5 min
80000
70000
60000
50000
40000
30000
20000
10000
0
GWT
#3
#4
#8
#1
#4
#19
p35S::LUP5
pLUP5::LUP5
b
Monodesmosidic oleanolic acid (with 2 hexose) peak area
11.0 min
14000
12000
10000
8000
6000
4000
2000
0
c
Monodesmosidic oleanolic acid (with 3 hexose) peak area
10.7 min
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0

310

311 Fig. 4 Expression of G-type *LUP5* gene increased glycosylated oleanolic acid and
312 hederagenin levels in G-type in G-type *B. vulgaris*. (a) Monodesmosidic hederagenin (with
313 3 hexose) peak area (EIC: m/z = 1003.5115 ± 0.2). (b) Monodesmosidic oleanolic acid (with
314 two hexoses) peak area (EIC: m/z = 617.4059 ± 0.2). (c) Monodesmosidic oleanolic acid
315 (with 3 hexose) peak area (EIC: m/z = 987.517 ± 0.2). GWT, wildtype G-type *B. vulgaris*;
316 p35S::LUP5, p35S::LUP5 transformed G-type *B. vulgaris*; pLUP5::LUP5, pLUP5::LUP5
317 transformed G-type *B. vulgaris*. Statistical significance is indicated by *($p<0.5$), ** ($p < 0.05$)
318 and *** ($p < 0.005$).

319 In conclusion, stable transformation of G-type *LUP5* in P-type significantly increased
320 one oleanolic acid monoglucoside (with one hexose) and three hederagenin-derived
321 monodesmosidic saponins (each with two hexoses) (Fig. 3a,b). Notably, the oleanolic acid
322 monoglucoside—eluting at 9.5 minutes—was detected exclusively in the transformed P-
323 type (Fig. 3b, Table S5). The most abundant hederagenin-derived saponin in P-type eluted
324 at 11.5 min, whereas hederagenin cellobioside (eluted at 12.3 min) remained the
325 dominant saponin in G-type (Fig. 3a, Table S6, Table S8). Introduction of G-type *LUP5* in G-
326 type slightly increased saponins, but their concentrations remained low compared to
327 other oleanolic acid- and hederagenin-derived compounds (Fig. 4a-c, Table S8).
328 Expresseion of G-type *LUP5* by its native promoter resulted in higher transformation
329 efficiency and induced greater accumulation of β-amyrin-derived sapogenins and their
330 glycosylated saponins compared to the 35S promoter (Fig. 1b, Fig. 3a-c, Fig. 4a-c, Table
331 S7).

332 ***CYP72A552* silencing decreased hederagenin derived saponin accumulation in G-type**
333 ***B. vulgaris***

334 To evaluate the impact of G-type *CYP72A552* silencing on insect resistance, a choice
335 insect feeding assay was conducted as described above. However, neither the transgenic
336 G-type nor the wild-type G-type plants were consumed by diamondback moth larvae (Fig.
337 5g). To assess the impact of *CYP72A552* downregulation on saponin and sapogenin levels
338 in G-type *B. vulgaris*, a metabolomic analysis was performed as described above. Silencing

339 of *CYP72A552* reduced the accumulation of six saponins in the G-type, including
340 hederagenin monoglucoside and three other hederagenin-derived monodesmosidic
341 saponins (each with two hexoses), monodesmosidic gypsogenin (with two hexoses), and
342 one unknown saponin (Fig. 5a-e). Based on MS/MS analysis, the saponin has a sapogenin
343 with a molecular weight of 457 m/z, along with one hexose and two methylpentoses.
344 Twenty-three other saponins, including seven glycosylated oleanolic acids, remained
345 unchanged (Table S9). The sapogenin levels from *CYP72A552* silenced line #13 and
346 wildtype G-type *B. vulgarism were* compared. Total hederagenin levels decreased by 40%,
347 while total and free oleanolic acid levels remained unchanged, in agreement with the
348 saponin data (Fig. 5a-f). Lupeol and β-amyrin remained undetectable in G-type *B. vulgaris*
349 (Table S7). In conclusion, silencing *CYP72A552* significantly reduced the biosynthesis of
350 hederagenin and its glycosylated derivatives in G-type *B. vulgaris*.

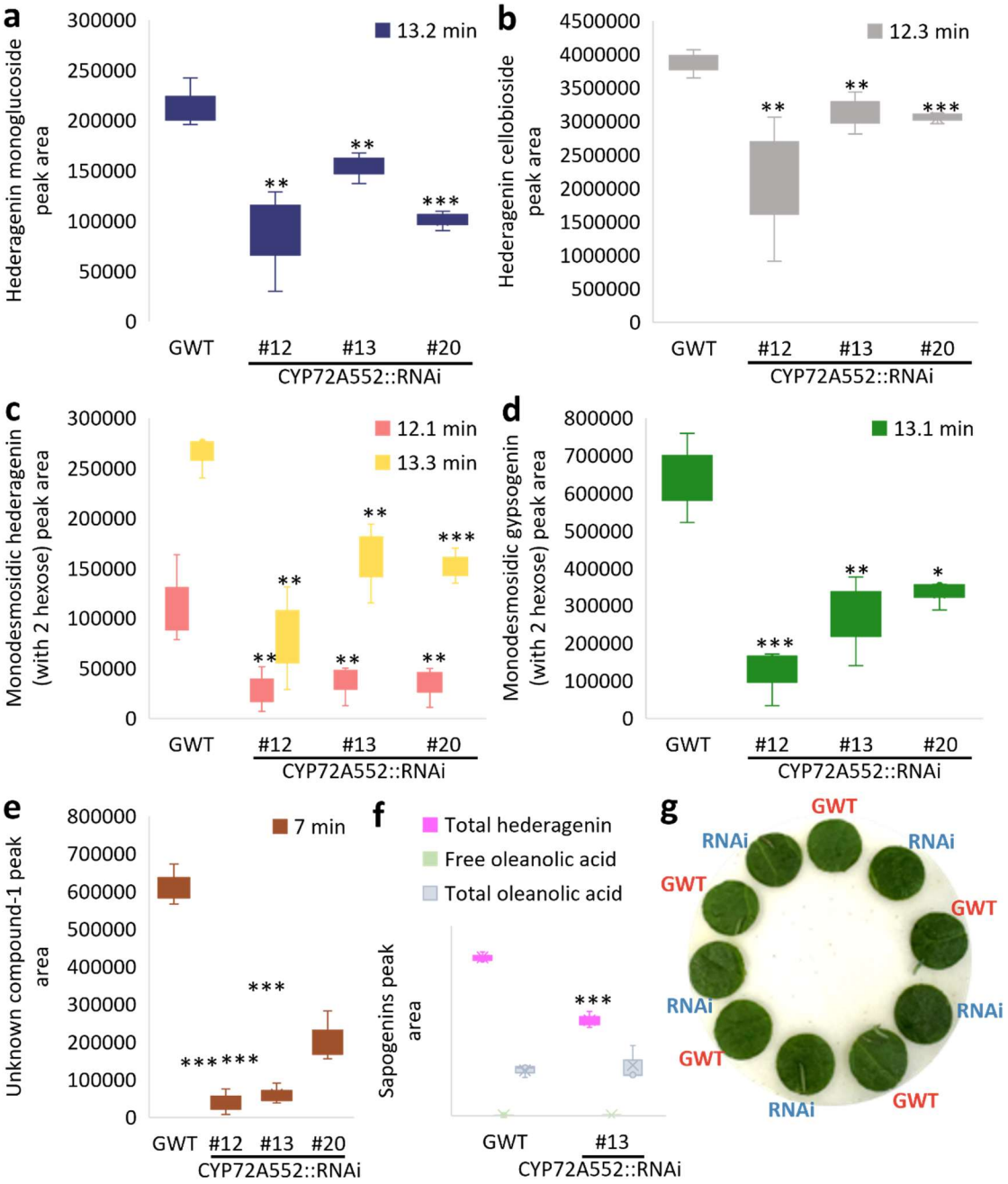


Fig. 5 Silencing *CYP72A552* decreased hederagenin and its derived saponins in G-type *B. vulgaris*. (a) Hederagenin monoglucoside peak area (EIC: m/z = 679.4063 ± 0.2). (b) Hederagenin cellobioside peak area (EIC: m/z = 841.4591 ± 0.2). (c) Monodesmosidic hederagenin (with two hexoses) peak area (EIC: m/z = 841.4591 ± 0.2). (d) Monodesmosidic gypsogenin (with two hexoses) peak area (EIC: m/z = 839.4409 ± 0.2).

357 (e) unknown compound-1 peak area (EIC: m/z = 901.2429 ± 0.2). (f) Peak area of
358 sapogenins, including total hederagenin (EIC: m/z = 437.3425 ± 0.2), free and oleanolic
359 acid (EIC: m/z = 439.3593± 0.2). GWT, wildtype P-type *B. vulgaris*; CYP72A552::RNAi,
360 *CYP72A552* silenced G-type *B. vulgaris*. Statistical significance is indicated by *(p<0.5), **
361 (p < 0.05) and *** (p < 0.005).

362 **Discussion**

363 Triterpenoid saponins are key mediators of plant–insect interactions and have
364 attracted attention due to their structure-dependent bioactivity and unique modes of
365 action. Key factors influencing insect resistance include saponin structure, composition of
366 the sapogenin, the types of sugar moieties, the length and number of glycoside chains,
367 and the glycosylation sites (Gao et al., 2011; Liu et al., 2019; Tian et al., 2021). Prior
368 metabolic engineering efforts have focused on modifying saponin composition by
369 disrupting biosynthetic genes in legumes (Confalonieri et al., 2021; Hodgins et al., 2024).
370 Confalonieri *et al*. (2021) knocked out *CYP93E2*, which hydroxylates β-amyrin at the C-24
371 position, in *Medicago truncatula*, resulting in transgenic lines that no longer produced
372 non-hemolytic soyasapogenols saponins but instead redirecting metabolic flux toward
373 the synthesis of hemolytic saponins. Hodgins *et al*. (2024) knocked out β-amyrin synthase
374 *BAS1* in pea (*Pisum sativum*), leading to a 99.8% reduction in saponin content in the seeds.
375 However, these approaches have not addressed how targeted saponin engineering
376 influences ecological traits such as insect resistance or consumption.

377 Here, we metabolically engineered the saponin biosynthetic pathway in the wild
378 crucifer *Barbarea vulgaris* to test whether specific triterpenoid profiles confer resistance
379 to insect herbivory. We have previously hypothesized—based on quantitative trait loci
380 analysis and insect assays with saponin-treated leaf disks—that differences in *LUP5*
381 expression between G- and P-type *B. vulgaris* underlie their distinct saponin profiles and
382 insect resistance (Augustin et al., 2012; Khakimov et al., 2015; Nielsen et al., 2010; Kuzina
383 et al., 2011). Our engineering strategy confirms that the susceptibility of the P-type to
384 diamondback moth feeding is primarily due to its lack of *LUP5* expression. LUP5 encodes

385 an oxidosqualene cyclase that produces β-amyrin—the precursor of oleanolic acid and
386 hederagenin-derived saponins—both implicated in insect deterrence. Interestingly, our
387 data suggest that the glycoside linkage has less impact on feeding behavior than the
388 triterpenoid backbone itself.

389 These findings confirm our earlier work showing that saponin toxicity is influenced
390 by both backbone structure and glycosylation pattern, particularly when hydroxylation
391 occurs at C23 and glycosylation at C3 (Augustin et al., 2012; Liu et al., 2019). Insect-
392 susceptible P-type plants predominantly produce lupeol-derived saponins, reflecting their
393 higher expression of LUP2, while G-type plants express *LUP5* and accumulate β-amyrin-
394 derived saponins, including oleanolic acid and hederagenin cellobioside—both
395 contributing to insect resistance (Augustin et al., 2012; Khakimov et al., 2015; Liu et al.,
396 2019). By combining gene transfer with ecological assays, our study provides a framework
397 for linking specialized metabolism with ecological function in a non-model plant system.

398 To test this hypothesis in an *in planta* system, the β-amyrin synthase *LUP5* gene was
399 expressed in both the insect resistant G-type and insect susceptible P-type *B. vulgaris*. In
400 the otherwise insect susceptible P-type, this resulted in up to a 95% reduction in leaf
401 consumption and increased levels of three hederagenin-derived monodesmosidic
402 saponins (each with two hexoses) and one oleanolic acid monoglucoside (Fig. 2a, Fig.
403 3a,b). In wildtype *B. vulgaris* G-type, hederagenin cellobioside is the predominant
404 saponin, while only small amounts of the other two hederagenin-derived saponins were
405 detected at retention times of 10.7 and 11.5 min (Fig. 4a,b, Table S8). In contrast, in the
406 *LUP5*-transformed P-type, the monodesmosidic hederagenin eluting at 11.5 min is more
407 abundant than the other two (Fig. 3a). This variation highlights differences in saponin
408 glycosylation patterns between P- and G-types, suggesting that these three hederagenin-
409 derived monodesmosidic saponins, particularly the one detected at 11.5 min, may
410 contribute to insect resistance in the transformed P-type plants. Hederagenin 3-*O*-
411 glucoside exhibits seven-fold stronger feeding reduction to diamondback moth and
412 tobacco hornworm compared to oleanolic acid 3-*O*-glucoside (Liu et al., 2019). This

413 enhanced efficacy has been linked to C-23 hydroxylation in hederagenin, which causes
414 the C-3 glucose moiety to adopt a different orientation relative to the sapogenin
415 backbone compared to oleanolic acid (Cárdenas, Almeida and Bak, 2019; Liu et al., 2019).
416 Considering the bioactivity and the relatively low increase of oleanolic acid
417 monoglucoside compared to hederagenin-derived monodesmosidic saponins in *LUP5*-
418 transformed P-type suggest that there is a CYP72A552 ortholog in the P-type that
419 effectively transforms the oleanolic acid to hederagenin, which then is glycosylated by
420 different UGTs than in the G-type *B. vulgaris* to other linkage types than the 1→4 linkage
421 cellobiosides that predominantly accumulate in the G-type.

422 Based on our findings, LUP5 is confirmed as the key determinant for insect resistance
423 differences between G- and P-type *B. vulgaris*. Furthermore, three hederagenin-derived
424 monodesmosidic saponins (each with two hexoses) may play a role in insect resistance in
425 *LUP5*-transformed P-type, particularly the most abundant saponin at retention time 11.5
426 min. Future work comparing the insect resistance differences between the elucidated
427 structures of these three hederagenin-derived monodesmosidic saponins could provide
428 further insights into how glycosylation patterns affect saponin bioactivity. Glycoside
429 linkage impacts cytotoxicity of saponins in *in vivo* assays (Chwalek et al., 2006). However,
430 how glycoside linkage affects insect resistance remains unknown. Unfortunately, due to
431 their low concentration, isolating and purifying these compounds for bioactivity testing
432 remains challenging. Future research should focus on identifying candidate
433 glycosyltransferases that produces a variety of glycoside linkage with the aim to produce
434 defense-related hederagenin-derived monodesmosidic saponins in planta.

435 Interestingly, when *LUP5* expression was driven by the constitutive 35S promoter,
436 *LUP5* was expressed at approximately sixteen-fold higher levels than in the best
437 *pLUP5::LUP5* transgenic line and also exceeded expression levels in both wild-type P- and
438 G-type plants (Fig. 2c). However, this high expression did not translate into significant
439 increased saponin production (Fig. 2a,b, Fig. 4a-c). In contrast, using the native *LUP5*
440 promoter not only resulted in about two-fold higher transformation efficiency (Fig. 1b)

441 but also led to greater accumulation of hederagenin and its glycosides in the P-type
442 background despite the overall much lower expression profile (Fig. 3a,c). These results
443 underscore that promoter choice critically influences metabolic output—not simply
444 through expression level, but likely through coordinated regulation aligned with
445 downstream biosynthetic processes.

446 While constitutive promoters like 35S often used to drive high transgene expression,
447 they can also impose metabolic burdens and reduce transformation efficiency due to
448 unintended gene expression as shown above. Studies show that although 35S enhances
449 transcript levels higher than native promoters, this does not always result in higher
450 protein accumulation or improved physiological performance than native promoter
451 (Seger et al., 2009; Beringer et al., 2017; Waller et al., 2023). Instead, its broad tissue
452 activity can lead to premature senescence, metabolic depletion, and reduced plant
453 productivity (Seger et al., 2009). Comparative analyses further reveal that native
454 promoters often outperform constitutive promoters in overall plant viability. For
455 example, in maize (*Zea mays*), the *Zm*Ubi1 promoter yielded higher transformation
456 efficiency and better tissue culture survival than the 35S promoter, despite lower
457 expression levels (Beringer et al., 2017). Similarly, *Anthoceros agrestis* expressing
458 *hygromycin resistance* gene under 35S showed growth retardation and smaller leaves,
459 whereas the *Aa*Ef1a promoter avoided these issues (Waller et al., 2023). These examples
460 highlight the value of tissue-specific expression, which prevents unnecessary metabolic
461 drain from non-target tissues and supports healthier development.

462 In conclusion, although the 35S promoter often drives high levels of transgene
463 expression, its constitutive activity across most tissues can lead to unintended metabolic
464 imbalances, reduced transformation efficiency, and pleiotropic effects on plant
465 development. Furthermore, expression in tissues where the gene is not biologically
466 relevant may not contribute to the desired function. In contrast, native promoters limit
467 gene expression to appropriate tissues and developmental stages, enabling more precise

468 metabolic control and optimized biosynthetic pathway regulation—factors that better
469 support the intended biological outcome.

470 The tissue-specific expression of *LUP5* appears to play a significant role in regulating
471 the accumulation of hederagenin and its glycosylated saponins in *B. vulgaris*. We
472 hypothesize that under the 35S promoter, *LUP5* is expressed throughout all leaf tissues,
473 potentially causing excessive β-amyrin accumulation and contributing to the observed
474 reduction in transformation efficiency. However, β-amyrin was undetectable in both wild-
475 type and transgenic *B. vulgaris*, possibly due to rapid metabolic conversion. To further
476 advance understanding of saponin biosynthesis in *B. vulgaris*, future studies should aim
477 to identify *LUP5* promoter-driven expression patterns at the tissue level, providing deeper
478 insight into the spatial regulation of saponin production.

479 We had anticipated that silencing of *CYP72A552* in G-type would reduce insect
480 consumption as hederagenin derived saponins are more bioactive than those derived
481 from oleanolic acid. However, our results show that while silencing of *CYP72A552*
482 decreased hederagenin biosynthesis by up to 40%, leading to a significant reduction in
483 hederagenin- and gypsogenin (which is further oxidized at C-23 to the ketone)- derived
484 monodesmosidic saponins. Specifically, the bioactive hederagenin monoglucoside,
485 hederagenin cellobioside and monodesmosidic gypsogenin (with two hexoses) were
486 decreased (Fig. 5a-d). The decrease in monodesmosidic gypsogenin may have been
487 caused by the reduced hederagenin biosynthesis or off-target effects (Fig. S2), possibly
488 resulting from the high nucleotide sequence identity of eight tandemly repeated *CYP72A*
489 homologs (Liu et al., 2019). Previous studies indicate that cellobiosides of hederagenin
490 and gypsogenin correlate with insect resistance in *B. vulgaris* in a concentration-
491 dependent manner (Shinoda et al., 2002; Agerbirk et al., 2003; Kuzina et al., 2009; Liu et
492 al., 2019). The hederagenin cellobioside concentration in G-type *B. vulgaris* leaves is
493 ~eleven-fold higher than the ED50 (50% effective dose) for *P. xylostella* (Shinoda et al.,
494 2002; Liu et al., 2019). Thus, even with a 40% reduction, the remaining saponin levels still
495 exceed the toxicity threshold required to deter feeding. Likewise, *LUP5*-transformed P-

496 type plants did not reach the full resistance potential of G-type plants, likely due to
497 incomplete accumulation of the most potent hederagenin saponins (Fig. 2a,b, Fig. 3a-c,
498 Fig. 5a-g). Overexpression of *LUP5* in G-type plants produced only marginal increases in
499 minor oleanolic acid and hederagenin saponins, suggesting that G-type plants may
500 already accumulate saponin at or close the acceptable threshold for accumulation (Fig.
501 5a-c, Table S8).

502 **Conclusion**

503 In summary, this study demonstrates that targeted metabolic engineering of
504 triterpenoid saponins in the wild crucifer *Barbarea vulgaris* is a robust approach to dissect
505 the ecological roles of structurally specific saponins and enhance insect resistance.
506 Expression of the β-amyrin synthase *LUP5* under its native promoter was more effective
507 than constitutive expression, leading to higher transformation efficiency and increased
508 accumulation of insect-deterring hederagenin-derived monodesmosidic saponins. This
509 underscores the importance of promoter choice and precise transcriptional regulation in
510 optimizing specialized metabolite production.

511 Our findings establish metabolic engineering as not only a tool for enhancing
512 resistance traits but also as a powerful approach for functional genomics. We identified
513 LUP5 as a crucial determinant of insect resistance in *B. vulgaris*, and showed that three
514 specific hederagenin-derived monodesmosidic saponins, each bearing two hexoses, are
515 associated for this defense.

516 Taken together, this work introduces a novel application of metabolic engineering to
517 unravel ecological functions of plant metabolites and offers promising strategies for crop
518 protection through fine-tuned biosynthetic pathway manipulation.

519

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Conflicts of interest

Authors declare that there are no conflicts of interest.

Author contributions

JS, PC, and SB designed the research and wrote the paper; JS and PC performed the research; JS, PC, JG and SB analysed the data; JG provided the data analysis strategy and standards.

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