Title: Genetic basis and selection of glyceollin induction in wild soybean

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Summary

- Glyceollins, a family of phytoalexin induced in legume species, play essential roles in responding to environmental stresses and in human health. However, little is known about the genetic basis and selection of glyceollin induction.
- We employed a metabolite-based genome-wide association (mGWA) approach to identify candidate genes involved in glyceollin induction from genetically diverse and understudied wild soybeans subjected to soybean cyst nematode stress.
- Eight SNPs on chromosomes 3, 9, 13, 15, and 20 showed significant association with glyceollin induction. Six genes close to one of the significant SNPs (ss715603454) on chromosome 9 fell into two clusters, and they encode enzymes in the glycosyltransferase class within the phenylpropanoid pathway. Transcription factors (TFs) genes, such as *MYB* and *WRKY* were also found within the linkage disequilibrium of the significant SNPs on chromosome 9. Epistasis and a strong selection signal were detected on the four significant SNPs on chromosome 9.
- Gene clusters and transcription factors may play important roles in regulating glyceollin induction in wild soybeans. Additionally, as major evolutionary factors, epistatic interactions and selection may influence glyceollin variation in natural populations.

Keywords

Epistasis, Gene cluster, mGWAS, phytoalexin, Plant and human health, Selection, Transcription factors, Wild soybean.

Abbreviations list:

bp	base pair
BLINK	bayesian-information and linkage-disequilibrium iteratively nested keyway
dpi	days post infection

FDR	false discovery rate
Fig. (Figs)	figure (figures)
LD	linkage disequilibrium
LOD	logarithm of the odds
Mbp	megabase pair
mGWAS	metabolite-based genome-wide association study
SNP	single nucleotide polymorphism
µm	micromolar
µg/g	microgram/gram

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55 **Introduction**

56 Plants produce diverse specialized metabolites (also known as secondary metabolites or
57 phytochemicals), which play a vital role in adapting to changing environments. Phytoalexins are
58 specialized metabolites synthesized *de novo* in response to various biotic and abiotic stresses.
59 Examples include indole alkaloid camalexin in *Arabidopsis,* phenolic aldehyde gossypol in cotton,
60 phenylpropanoid stilbenes in grapevines, isoflavonoid-derived glyceollins in legume, and
61 momilactones and phytocassanes terpenoids in rice (Donnez et al., 2011, Jahan et al., 2019, Jeandet
62 et al., 2002, Jeandet et al., 2020, Saga et al., 2012, Wang et al., 2009, Yamamura et al., 2015).
63 Isoflavonoids have become a research hot spot due to their various pharmacological properties and
64 essential roles in plant defense. The major isoflavones in soybeans are genistein, daidzein, and
65 glycitein, and they make up about 50%, 40%, and 10%, respectively, of the total isoflavone content.
66 Trace amounts of glyceollins are induced transiently with abiotic and biotic stresses (Jahan et al.,
67 2019, Subramanian et al., 2006). They have multiple effects, including fostering symbiosis
68 between soybean and *Bradyrhizobium japonicum* and inhibiting the growth of various microbes
69 (Graham and Graham, 1996, Subramanian et al., 2006). Moreover, they have anti-cancer,
70 antioxidant, and neuroprotective properties (Bamji and Corbitt, 2017, Kim et al., 2012,
71 Nwachukwu et al., 2013, Seo et al., 2018). However, studies on glyceollins are mainly focused on
72 their medicinal properties, while little is known about how their induction is regulated.

73

74 Phytoalexins have been considered the target of natural selection due to their activities in biotic
75 and abiotic stress responses in natural environments (Miyamoto et al., 2016, Pichersky and Gang,
76 2000, Qi et al., 2004). Therefore, in our study, we chose wild soybean (*Glycine soja*), a wild
77 relative of soybean (*Glycine max)*, to delineate genetic basis and evolution of glyceollin
78 accumulation resulting from biotic stress, i.e., soybean cyst nematode (SCN), the most devastating
79 soybean pest worldwide (Tylka and Marett, 2021). Wild soybeans thrive in diverse habitats and
80 harbor much higher, underexplored genetic diversity than cultivated soybean (Zhang et al., 2019).
81 Hence, it is an ideal system to understand the genetic basis and evolution of glyceollin variation.
82 Eventually, the essential genes identified in wild soybean can be used for metabolic engineering
83 or in a breeding program to develop nutrition-rich biofortified soybean cultivars as they exhibit
84 similar genome size and content with small reproductive isolation (Singh and Hymowitz, 1999).

85

86 A metabolic gene cluster is a group of (two or more) genomically co-localized and potentially
87 coregulated non-homologous genes that encode enzymes involved in a particular metabolic
88 pathway (Nützmann et al., 2016, Töpfer et al., 2017). They have been a common phenomenon
89 since the early days of microbial genetics (Koonin, 2009, Rocha, 2008, Zheng et al., 2002).
90 However, gene clusters in plant metabolic pathways have been discovered only recently, even
91 though microbes and plants are both extremely rich sources of metabolic diversity. A study by
92 Chae et al. (2014) on metabolic gene clusters in *Arabidopsis,* soybean, sorghum, and rice suggested
93 that approximately one-third of all the metabolic genes in *Arabidopsis,* soybean, and sorghum, and
94 one-fifth in rice were rich in gene clusters across primary and specialized metabolic pathways
95 (Chae et al., 2014). There is compelling evidence indicating that the highly plastic plant genome
96 itself generates metabolic gene clusters via gene duplication, neofunctionalization, divergence, and
97 genome reorganization instead of horizontal gene transfer from microbes (Osbourn and Field,
98 2009). This suggests that plants rewire their genome to gain new adaptive functions driven by the
99 need to survive in distinct environments. Systematic mining and functional validation of the
100 candidate genes in such clusters will facilitate the discovery of new enzymes and chemistries that
101 render pathway prediction. Moreover, metabolic gene clusters are likely to be located within
102 dynamic chromosomal regions, and thus, many identified so far may be due to recent evolution
103 (Field et al., 2011, Matsuba et al., 2013, Qi et al., 2004). If so, investigation of these clusters can
104 provide insights into their evolutionary history. The vast and diverse array of specialized
105 metabolites that are produced through multi-step metabolic pathways play an important role in
106 plant adaptation to various ecological niches. However, the occurrence, prevalence, and evolution
107 of such gene clusters in plants are largely unknown. Thus, the study of plant metabolic gene
108 clusters has implications for molecular biology and evolutionary genomics (Chavali and Rhee,
109 2018, Nützmann et al., 2016, Takos and Rook, 2012, Yeaman and Whitlock, 2011).

110 Due to the extraordinary metabolic diversity, to date, less than 50 plant-specialized metabolic
111 pathways have been biochemically and genetically identified (Nützmann et al., 2016).
112 Metabolomic GWAS (mGWAS) offers an effective approach to understand the genetic basis of
113 metabolites and their associated traits (Chan et al., 2010, Chan et al., 2011, Luo, 2015,
114 Riedelsheimer et al., 2012). mGWAS allows the identification of common polymorphic regions
115 controlling complex metabolic traits by substantially increasing association panel and genome-

116 wide molecular markers. Besides elucidating genetic architecture, mGWAS can also be used to
117 infer gene functions (Luo, 2015). Hence, mGWAS provides a comprehensive approach to
118 discovering candidate genes. Thus far, it has been used to uncover the genetic basis of variations
119 of a number of different metabolites. For example, Chen et al. (2014) carried out a rice mGWAS
120 study that identified 36 candidate genes influencing the variation of metabolites with physiological
121 and nutritional importance (Chen et al., 2014).

122

123 The isoflavonoid pathway has been relatively well studied (Sukumaran et al., 2018). However, it
124 is still not clear how glyceollin induction is regulated. This study is the first to employ genomic
125 and evolutionary approaches to understand the genetic basis and selection of glyceollin induction.
126 Our study provides a fundamental basis for the long-term goal of developing glyceollin-fortified
127 soybean cultivars that would improve plant and human health to meet current and future global
128 challenges. In this study, we aim to address these three questions: (1) What is the genetic basis of
129 variation in glyceollin induction by SCN? (2) Are there any gene clusters and transcription factors
130 involved in glyceollin variation? (3) Are epistatic interactions and natural selection important
131 evolutionary factors influencing the variation of glyceollin induction?

132

133 **Materials and Methods**

134 **Plant materials**

135 A total of 264 accessions of wild soybean, *Glycine soja,* from a wide geographic range, originally
136 collected from China, Japan, Russia, and South Korea, were utilized (Table S1). The seeds of these
137 ecotypes were obtained from the USDA national germplasm resources laboratory
138 (https://www.ars-grin.gov/).

139

140 **Plant preparation, SCN inoculation, and sample collection**

141 Seed preparation, germination, transplanting, and soybean cyst nematode (SCN, *Heterodera*
142 *glycines Ichinohe*, HG type 1.2.5.7) inoculation were performed following a previously developed
143 protocol (Zhang et al., 2017a, Zhang et al., 2017b, Zhang and Song, 2017). Whole root tissues
144 were collected and weighed five days post-infection (dpi). The 5 dpi time point was chosen because

our previous study suggested a significant inhibition in SCN development in a resistant genotype compared to normal growth in a susceptible genotype (Zhang et al., 2017a, Zhang et al., 2017b). All samples were flash frozen in liquid nitrogen and stored at -80 °C. Four biological replicates per wild soybean genotype were used, eventually a total of 1,020 samples.

Metabolite extraction and quantification

We employed the extraction method of metabolites from root tissue described in Strauch et al., (2015). The metabolite profiling was provided by the service from David H. Murdock Research Institute at the North Carolina Research Campus. Peaks that were consistently detected in at least three biological replicates within each genotype were used for downstream analyses. Each metabolite was confirmed using pure standard compounds, including daidzein, daidzein-d6, and glyceollin. Due to the low concentrations of these compounds and the small sample masses of the wild soybean root samples that had been collected, we used a signal-to-noise ratio of $\geqslant 10$ for the measurement of the peaks for glyceollin and daidzein. Our method successfully measured daidzein (μg/g root) and glyceollin (unitless) in 264 accessions of wild soybean *G. soja* roots quantitatively and semi-quantitatively, respectively. Following method development, optimization, and analyses of the test samples, calibration curves were designed using at least six different concentrations of daidzein, created in triplicate to quantify known concentrations of daidzein and glyceollin. A second-degree polynomial was derived from the known concentrations of the standard curve samples and the mass spectrometer response (daidzein/internal standard) from the standard curve data. The resulting polynomial was used to calculate the concentrations of daidzein in the experimental samples. Low, medium, and high QC (quality control) samples were created to assess the accuracy of the calculations. We used the ratio of glyceollin (unitless) to daidzein (μg/g root) (GVSD) as our phenotypic trait. This phenotype henceforth is denoted GVSD.

Genotypic data

Genotype data for the 264 accessions were obtained from SoySNP50K (Song et al., 2013), which included 32,976 genome-wide single nucleotide polymorphic markers (SNPs) with a minor allele frequency (MAF) of at least 5%.

174 **Metabolite-based genome-wide association study (mGWAS) and linkage disequilibrium**
175 **estimation**

176 Our genome-wide association analysis was conducted on GVSD (a ratio of glyceollin mean to
177 daidzein mean) in response to SCN infection on all 264 ecotypes using the BLINK algorithm
178 implemented in the GAPIT R package (2.0) (Tang et al., 2016). To minimize false-positive
179 associations, we controlled population structure among genotypes with four principal components.
180 Heritability estimate and SNP effect were calculated by running GWAS applying CMLM and
181 MLM methods, respectively, implemented in the GAPIT R package (2.0) (Tang et al., 2016).

182

183 A conventional Manhattan plot was generated using the qqman R package to visualize the SNPs
184 (Turner, 2014). In addition to the genome-wide significant threshold, we also calculated the
185 chromosome-wide Bonferroni thresholds using independent SNPs estimated on each chromosome
186 following the method of Li and Ji (2005) (Li and Ji, 2005). Linkage disequilibrium (LD) was
187 calculated across the panel with the TASSEL program, version 5 [6], for the significant SNPs
188 identified from the GWAS analysis. LD was measured using squared correlation R-squared (r^2) of
189 0.2 (upper right in the LD plot) and p-value < 0.05 (the lower left in the LD plot). A pairwise LD
190 was generated following the R function described by Shin et al. (2006) (Shin et al., 2006). Genes
191 within LD blocks containing significant SNPs were identified as potential sources of candidates
192 for further analyses.

193

194 **Identification of candidate genes**

195 For extensive gene mining of our identified gene pool, we used an array of bioinformatics tools.
196 Such an approach can improve the accuracy of candidate gene and gene cluster predictions and
197 resolve inconsistencies among the bioinformatics tools (Chavali and Rhee, 2018). Specifically, a
198 pairwise linkage disequilibrium (LD) analysis was initially used for potential candidate gene
199 identification. Then, genes in each LD block were examined as potential candidate genes, and their
200 annotations were obtained from the Phytozome v13 database (Goodstein et al., 2011). Afterward,
201 a GO enrichment analysis of the identified candidate genes was performed using ShinyGO v0.66:
202 Gene Ontology Enrichment Analysis (p-value cutoff (FDR, false discovery rate) = 0.05) (Ge et al.,

203 2020), Soybase GO Enrichment Data (Grant et al., 2010). To investigate the involvement of these
204 potential candidate genes in metabolic pathways, a database search was performed through an
205 annotation file from Phytozome v13 (Goodstein et al., 2011), Soybase (Grant et al., 2010), SoyCyc
206 10.0 Soybean Metabolic Pathway (Hawkins et al., 2021), and Pathview databases (Luo et al., 2017).
207 Finally, a PMN plant metabolic cluster viewer was applied to categorize enzymes into classes
208 (signature or tailoring) and metabolic domains (Hawkins et al., 2021).

209

210 **Analysis of epistatic interactions**

211 For any significant SNPs uncovered in the GWAS analysis, it is useful to test whether, beyond
212 their direct effects, they also exhibited interactive effects on GVSD. To accomplish this, we first
213 produced numerically formatted genotypes, in which the homozygous genotype index value is 1
214 and -1 and the heterozygous 0. This allows us to test for epistasis for each pairwise combination
215 in a simple general linear model with 1 degree of freedom for the additive effects of each of the
216 two SNPs and their interaction. We included the first four principal components from the GAPIT
217 analysis in the model to be consistent with the GWAS scan, where these components were used to
218 adjust for structural relatedness (see below). The significance of all interactions was evaluated with
219 the sequential Bonferroni procedure. To illustrate the interactions of SNP pairs, we also calculated
220 regressions of GVSD on each SNP, but at each of the three genotypes (using the -1, 0, and 1 index
221 values) of the second SNP involved in the significant interaction.

222

223 **Extended haplotype homozygosity analyses**

224 To test allele-specific selection patterns of the identified significant SNPs, we analyzed extended
225 haplotype homozygosity (EHH, (Sabeti et al., 2002)) for each significant SNP. The EHH analysis
226 was conducted in SELSCAN v.1.2.0a (Szpiech and Hernandez, 2014) with default parameters, and
227 only SNPs with MAF > 0.05 was used in this analysis.

228 **Results**

229 **Genomic dissection of glyceollin accumulation upon biotic induction**

230 We identified a total of eight significant SNPs, with four located on chromosome 9 and the others
231 on chromosomes 3, 13, 15, and 20 (Fig. **1a**, Table 1). These SNPs were identified based on both
232 genome-wide Bonferroni threshold of 5.104 and chromosome-wide Bonferroni thresholds that

233 varied narrowly from 3.79 to 3.82 among the 20 chromosomes (3.803 on chromosome 9) (Figs
234 **1a,b**, Table S2). The manhattan and Q-Q (quantile-quantile) plots are shown in Fig.**1a,b,c**. The
235 four significant SNPs on chromosome 9 are located close to each other within a 535 kb region
236 (Table S2). The broad-sense heritability (h^2) was estimated 35% (Table S2).

237 **Linkage disequilibrium analysis and candidate gene identification**

238 We identified a total of 666 possible candidate genes within the linkage disequilibrium (LD) blocks
239 of the eight significant SNPs (soybean reference genome *Glycine max* Wm82.a2.v1) (Goodstein
240 et al., 2011, Zhou et al., 2015). The LD block on chromosome 9 showed the strongest LD with a
241 long range compared to the others (Figs **2b**, **S1, S2**). We considered r^2>0.2 as a cutoff for our LD
242 analysis, where r^2 is the extent of allelic association between a pair of sites (Weir, 1990). Candidate
243 gene *Glyma.09G128200* shows the highest level of LD near the significant SNPs on chromosome
244 9 compared to the LD block for the rest of the significant SNPs on this chromosome (Figs **2b**, **S1**).
245 The functional annotation of the candidate genes within this block is biosynthetic enzymes
246 involved in isoflavonoid pathway, as well as regulatory genes such as *WRKY* and *MYB*
247 transcription factors (Tables 1, S3, and S4), which may indicate their transcriptional level
248 involvement in glyceollin induction in response to SCN stress (Colinas and Goossens, 2018).

249 We also found putative genes encoding enzymes involved in the specialized metabolic pathways
250 within the LD blocks of the significant SNPs on chromosomes 3, 13, 15, and 20. The enriched GO
251 category includes flavonoid biosynthesis pathway, phenylpropanoid metabolic process, linamarin
252 biosynthesis, and terpenoid biosynthesis (Table S5). Apart from the biosynthetic enzymes on these
253 chromosomes, we also found transcription factor genes, such as *WRKY*, *MYB*, and *NAC* (Table
254 S5).

255

256 **Metabolic gene clusters identification**

257 We were particularly interested in the candidate genes in the branch from daidzein to glyceollin in
258 the isoflavonoid biosynthesis pathway (Lozovaya et al., 2007). We found that the identified
259 candidate genes on chromosome 9 are clustered together, and they fell into two clusters. Both of
260 these two clusters belong to tailoring enzyme glycosyltransferase within phenylpropanoid
261 specialized metabolic domain. And six genes are within the branch of isoflavonoid biosynthesis

262 pathway. Two of these six genes, *Glyma.09G127200* and *Glyma.09G127300,* are called cluster 1,
263 while the rest four (*Glyma.09G127700*, *Glyma.09G128200*, *Glyma.09G128300*, and
264 *Glyma.09G128400*) are called cluster 2 (Table S3).
265
266 Further investigation of annotation of these candidate genes within the gene clusters (Table S4),
267 we found *Glyma.09G127200* gene encodes a glucosyltransferase that may act on 4'-methoxy
268 isoflavones biochanin A, formononetin, 4'-hydroxy isoflavones genistein, and daidzein substrates.
269 However, the enzyme does not act on isoflavanones, flavones, flavanones, flavanols, or coumarins
270 (Köster and Barz, 1981). Within the same cluster, *Glyma.09G127300* has similar annotations and
271 functions as *Glyma.09G127200*. Interestingly, the four genes within cluster 2 have a similar
272 functional annotation as *Glyma.09G127200 and Glyma.09G127300* in cluster 1, and all these four
273 genes encode isoenzymes (Table S4). Such a link between these two gene clusters indicates their
274 proximity in the metabolic pathway.
275
276 **Epistatic interactions among all significant SNPs**
277 The results of the epistasis tests for each of the 28 pairwise combinations of the eight significant
278 SNPs are shown in Table 2. Three probabilities, all associated with the SNP on chromosome 20,
279 were not estimable (Table 2). Among the remaining 25 SNP pairs, 20 show statistical significance.
280 Particularly noticeable is the high significance for all interactions of the SNPs on chromosomes 3,
281 13, and 15. Three of the six pairs among the four SNPs on chromosome 9, all involving
282 ss715603462, also are statistically significant. In general, therefore, this is evidence for substantial
283 epistasis among these SNPs affecting GVSD.
284
285 These epistatic interactions of the SNP pairs are illustrated in Fig. **3** for each of the four chosen
286 combinations. For example, in panel **a** (Fig. **3a**), it can be seen that regression slopes of GVSD on
287 ss715603454 are close to 0 for ss71585948 CC genotype but are positive for TC and especially
288 TT genotypes. In panel d (Fig. **3d**), regression slopes of GVSD on ss715603471 are negative for
289 ss715603462 AA and GA genotypes but positive for GG genotypes. With no epistasis, these slopes
290 would be expected to be roughly parallel, but in fact, they diverge considerably from parallelism
291 in these four examples.
292

293 **Significant SNPs exhibited extended haplotype homozygosity**

294 The extended homozygosity analysis (EHH) analyses revealed allele-specific EHH values of the
295 significant SNPs (ss715603454, ss715603455, ss715603462, and ss715603471) on chromosomes
296 9 (**Fig. 4**). For example, T allele of ss715603454 showed much higher EHH value than G allele.
297 Alleles of significant SNPs on the other chromosomes showed compatible EHH values (**Fig. 4**).
298

299 **Discussion**

300 **Metabolic gene clusters in glyceollin induction**

301 Gene clusters have been reported to play important roles in phytochemical diversity in *Arabidopsis*,
302 sorghum, soybean, and rice (Chae et al., 2014). However, their roles in regulating metabolic
303 variation in wild species are relatively less investigated. Even though the isoflavonoid biosynthesis
304 pathway is relatively well studied, the genetic regulation of glyceollin induction is unclear.
305 Particularly, the contribution, prevalence, and occurrence of gene clusters in plant metabolic
306 diversity are largely unclear. Our mGWAS results suggest there are two gene clusters with
307 functionally related but non-homologous genes, which may involve in glyceollin induction in wild
308 soybean. Thus far, these are the first reported plausible gene clusters involved in glyceollin
309 accumulation induced by biotic stimuli. These gene clusters suggest that glyceollin may be
310 synthesized where the enzyme-encoding genes are adjacent to each other on the same chromosome
311 (Chavali and Rhee, 2018). Physical clustering of genes with similar functions can facilitate co-
312 inheritance of alleles with favorable combinations and their coordinated regulations at chromatin
313 level (Chu et al., 2011, Osbourn, 2010a). Besides, such clusters incline to locate in the sub-
314 telomeric regions (Gierl and Frey, 2001, Qi et al., 2004, Sakamoto et al., 2004), near the ends of
315 chromosomes that are known to harbor mutations. For example, an examination of the complete
316 genome sequence revealed that the maize *DIMBOA* cluster is located close to the end of
317 chromosome 4 (Farman, 2007, Jonczyk et al., 2008). Thus, identifying the positions of the genes
318 can contribute to inferences of possible mechanisms underlying chemical diversity in natural
319 populations.
320

321 Tailoring enzymes, such as methyltransferases, glycosyltransferases, *CYPs*,
322 dehydrogenases/reductases, and acyltransferases are responsible for modifying the chemical
323 backbone of specialized metabolites (Osbourn, 2010b). The gene clusters we found are associated

with tailoring or regulating glycosyltransferase enzymes. A common defense mechanism of plants involves glycosylation of secondary metabolites by involving these enzymes (Mylona et al., 2008). Therefore, the clustering of the genes encoding glycosyltransferase on chromosome 9 indicates the formation of stress-induced (i.e., SCN stress in our study) protective compounds. For example, the cyclic hydroxamic acid *(DIBOA)* in maize (Frey et al., 1997, Gierl and Frey, 2001), the triterpene avenacin in oat (Field and Osbourn, 2008, Mugford et al., 2009, Qi et al., 2004, Qi et al., 2006), and two gene clusters associated with diterpene (momilactone and phytocassane) synthesis in rice, which may be pre-formed or synthesized after stress induction for plant defense. Disruption of such gene clusters may compromise pest and disease resistance and lead to the accumulation of toxic pathway intermediates (Chu et al., 2011). In the multi-step plant specialized metabolic pathways, rapid adaptation to a particular environmental niche could result in highly diverse and rapidly evolving metabolic gene clusters (Osbourn and Field, 2009). Hence, the level of conservation of the identified gene clusters in this study across different *Glycine soja* genotypes can shed light on evolutionary insight of these clusters (Field and Osbourn, 2008). Synthetic biology and functional genetics can further help investigate the organization and contribution of these clusters in metabolite diversity, as well as decipher the mechanism of adaptive evolution and genome plasticity (Chu et al., 2011, Osbourn, 2010b).

Plausible transcriptional factors in glyceollin induction

Advancement of genetics, genomics, and bioinformatic approaches facilitate the prediction and identification of a large number of genes, including transcription factors associated with plant-specialized metabolic pathways (Anarat-Cappillino and Sattely, 2014, Moore et al., 2019). However, the transcriptional regulators of specialized metabolism are less well characterized (Shoji and Yuan, 2021). The regulation of highly diverse plant specialized metabolic pathways is dynamic given the ever-changing environment. Such regulation generally occurs at transcription level, and thus, it requires coordinated regulation often mediated by transcription factors (TFs) (Colinas and Goossens, 2018, Shoji, 2019). For instance, *MYB* and basic helix-loop-helix *(bHLH)* TF family genes were reported to regulate anthocyanin and related flavonoid biosynthetic pathways in a wide range of species (Chezem and Clay, 2016). Moreover, significant modifications of these regulatory genes give rise to the vast diversity in plant specialized metabolism (Huang et al., 2018, Springer et al., 2019).

355

356 It is possible that transcription factors, such as *MYB* and *WRKY* TFs on chromosome 9, may
357 influence glyceollin induction. This indicates regulation of glyceollin induction with SCN stress
358 may involve a highly complex interplay among multiple genes and pathways. Previous studies
359 reported that gene families of transcription factors, such as *NAC*, *MYB*, *bHLH*, and *WRKY*,
360 exhibited conservative patterns among *Arabidopsis*, cotton, grapevine, maize, and rice (Ibraheem
361 et al., 2015, Ogawa et al., 2017, Saga et al., 2012, Xu et al., 2004, Yamamura et al., 2015, Zheng
362 et al., 2006). These plant species produce various phytoalexins, such as indole alkaloids, terpenoid
363 aldehydes, stilbenoids, deoxyanthocyanidins, and momilactones/ phytocassanes,
364 respectively. This gives rise to the question of whether these TFs are as diversified as the metabolic
365 pathways, or they maintain conservative patterns among species. The investigation of TFs binding
366 promoter regions can give insights if the pathways are co-opted into stress-inducible regulation by
367 the respective TFs (Jahan et al., 2019). The homology of TFs among different plant species can
368 help metabolic engineering a wide variety of crop plants to produce phytoalexins in greater
369 amounts.

370

371 In addition to enzyme-encoding genes, TF genes can also be found as gene clusters. For example,
372 the gene cluster of TF *ERF* (jasmonate (JA)- responsive ethylene response factor) consists of five
373 *ERF* genes in tomato (Cárdenas et al., 2016, Thagun et al., 2016), while eight in potato (Cárdenas
374 et al., 2016), two clusters of ten and five in tobacco (Kajikawa et al., 2017), five in *C. roseus* (Singh
375 et al., 2020), four in *Calotropis gigantea* (Singh et al., 2020), and four in *Glesemium sempervirens*
376 (Singh et al., 2020). Besides, TFs involved in plant specialized metabolism can be found in arrays
377 (Shoji and Yuan, 2021, Zhou et al., 2016). So, it is possible that the TFs we identified are located
378 in the same genomic neighborhood as arrays or biosynthetic gene clusters (BGCs). The co-
379 regulation hypothesis of gene clusters poses that clustering of TFs can help coregulate genes in a
380 pathway. Although co-regulation also exists between un-clustered metabolic pathways, clustering
381 may accelerate the recruitment of genes into a regulon (Smit and Lichman, 2022, Wisecaver et al.,
382 2017).

383

384 **Epistasis and plausible selection on glyceollin induction**

385 Metabolic traits have been reported to have low heritability due to environmental effects on their
386 accumulations (Rowe et al., 2008). Recent studies have shown strong epistatic interactions of
387 genes influencing variation of plant specialized metabolites, which may impact fitness in the field
388 (Brachi et al., 2015, Kerwin et al., 2015, Kerwin et al., 2017). For example, numerous epistatic
389 interactions influence the highly complex genetic architecture responsible for *Arabidopsis*
390 metabolism (Kliebenstein, 2001, Kliebenstein et al., 2001). Moreover, a mixture of positive and
391 negative epistatic interactions can help identify significant QTLs located within a biosynthetic
392 pathway (Rowe et al., 2008). Compared to expression regulations, the power of epistasis in
393 metabolomics is that they can better indicate the interconnectedness of metabolites within the
394 metabolic pathway (Arita, 2004, Fell and Wagner, 2000, Jeong et al., 2000). The widespread
395 interactive effects found among our identified significant SNPs affecting targeted metabolic traits
396 may be a consequence of the interconvertibility between daidzein and glyceollin.
397
398 Genes containing causal variation for plant defensive compounds may influence field fitness and
399 thus are likely under natural selection (Kroymann, 2011). For example, Benderoth et al. (2006)
400 detected positive selection in glucosinolate diversification in *Arabidopsis thaliana* and its relatives
401 (Benderoth et al., 2006). Prasad et al. (2012) showed positive selection for a mutation on a
402 metabolic pathway gene could enhance resistance to herbivory in natural populations of a rocky
403 mountain cress species (Prasad et al., 2012). We detected strong signals of selection on the SNPs
404 significantly associated with glyceollin phenotypes with EHH and LD analyses (Figs **4, 2b,** and
405 **S1**). For example, the LD surrounding the significant SNP ss715603454 that is next to the
406 identified gene clusters is more extensive, suggesting strong selection in this region (Figs **2b, S1**).
407 Meanwhile, the two alleles of this significant SNP, G and T, showed different EHH values, with
408 T exhibiting much longer haplotype homozygosity. This indicates that this T allele may be under
409 recent positive selection. Interestingly, the T allele is significantly associated with higher induction
410 of glyceollin and has a higher frequency in South Korea (Fig. **2c,d**). The allele-specific EHH
411 pattern and their geographic distribution may be due to heterogeneous selection pressure in nature.
412
413 **Perspectives and future directions of our study**
414 Plant specialized metabolites exhibit extreme quantitative and qualitative variation. Therefore,
415 high-throughput metabolite profiling, such as LC-MS analysis coupled with GWAS (as applied

416 here) can help better understand the genetic contributions to metabolic diversity in natural
417 populations. A common assumption is that biological variables or traits should show a normal
418 distribution, and skewed data may indicate measurement error. However, the scenario is different
419 in metabolomics, especially in secondary metabolism. For instance, a ratio of two related
420 compounds, rather than their separate values, may provide a comprehensive understanding of the
421 underlying enzymatic process (Byrne et al., 1996, Chan et al., 2011, Kliebenstein, 2001,
422 Kliebenstein et al., 2001, Kliebenstein, 2007, McMullen et al., 1998, Petersen et al., 2012, Prasad
423 et al., 2012, Yencho et al., 1998). We used a ratio of glyceollin and daidzein concentrations as the
424 phenotypic trait for our association study. The use of a metabolic ratio also may produce: (1) a
425 reduction in the variability of the data collected for the biological replicates and thus increase
426 statistical power and (2) a reduction in overall noise in the dataset by canceling out systemic
427 experimental errors. Most importantly for our purposes, the glyceollin to daidzein metabolite ratio
428 is correlated to the corresponding reaction rate under optimal steady-state assumptions, as this
429 metabolite pair is connected in the phenylpropanoid biosynthetic pathway (Petersen et al., 2012,
430 Suhre et al., 2011).
431
432 The natural world has a lot to offer in tackling diseases and global food scarcity. There is a need
433 to develop new medicines and future value-increased food by unlocking the uncharted gene pools
434 of wild plants. Our chosen study system crop wild relative of soybean poses much higher and
435 underexplored genetic diversity than its domesticated descendants. Given that glyceollin is
436 produced in trace amounts, it is an exciting challenge to define the plant metabolic gene clusters
437 and transcriptional regulators in the glyceollin biosynthesis pathway. Besides complex cancer
438 treatment and therapies, the rise of different types of tumors and tumor subtypes urges the need
439 for new drugs. Along with glyceollin's role in plant defense, it has been well-documented for anti-
440 cancer activities. Our follow-up studies will apply transcriptomics and functional validation of the
441 candidate genes, which can expand our focus to explore associations of genes in clusters to
442 understand their involvement in regulating glyceollin biosynthesis at the systems level. As
443 phytochemical variation can be caused by both structural genes and their expression differences,
444 it will be interesting to explore the role of pathway-specific regulators (i.e., transcription factors)
445 in glyceollin induction (Osbourn, 2010b). Our results suggest that improving our fundamental

446 knowledge of plant specialized metabolic gene clusters and regulators will facilitate metabolic
447 engineering with improved metabolic traits for sustainable agriculture and novel pharmaceuticals.
448

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460

461 **Author contributions**

462 B-H.S. conceived the study and designed the experiment. H.Z., J.W., R.R., C.B. and F.Y.
463 conducted the experiments and collected the data. F.Y., H.Z., L.L., and B.W. performed data
464 analysis. F.Y. drafted the original manuscript with input from . F.Y., H.Z., L.L., B.W., J.W. and
465 B-H.S. reviewed and edited the manuscript. All authors have read and agreed to the published
466 version of the manuscript.

467

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807 **Figure legends**

808 **Fig. 1** GWAS of Glyceollin induction with SCN stress: A genome-wide **(a)** and chromosome-
809 wide **(b)** Manhattan plots, with thresholds of 5.104 and 3.803, respectively; **(c)** quantile-quantile
810 (QQ) plot. Significant SNPs are found on chromosomes 3, 9, 13, 15 and 20 at a 5% genome-wide
811 threshold, the probability of 7.86×10^{-6} resulted in a threshold of 5.01 (solid red line in the genome-
812 wide Manhattan plot) **(a)**. The 5% chromosome-wide LOD threshold resulted in significant p-
813 values of 1.57×10^{-4} (threshold 3.803, solid blue line) **(b)**.

814

815 **Fig. 2** An LD decay measured as R square for pairwise markers and plotted against their distance
816 **(a)** and LD plot for chromosome 9 for significant SNPs. The black diagonal denotes LD between
817 each site and itself **(b)**. Geographic range of the alleles of significant SNPs close to the gene
818 clusters on chromosome 9 **(c)**. Allele frequency in each population. Allele frequency in different
819 geographic regions for a significant SNP was generated using JMP®, Version 15. SAS Institute
820 Inc., Cary, NC, 1989–2021. **(d)**.

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822 **Fig. 3** Epistatic interactions of the SNP pairs for each of four chosen combinations. Regression
823 slopes of GVSD on ss715603454 are close to 0 for ss715603454 CC genotypes but are positive
824 for TC and especially TT genotypes **(a)**. Regression slopes of GVSD on ss715603462 are close to
825 0 for ss715585948 CC genotypes but are negative for TC and especially TT genotypes **(b)**.
826 Regression slopes of GVSD on ss715615975 are close to 0 for ss715585948 TT genotypes but are
827 negative for TC and especially CC genotypes **(c)**. Regression slopes of GVSD on ss715603471
828 are negative in sign for ss715603462 AA and GA genotypes, but positive in sign for GG genotypes
829 **(d)**.

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831 **Fig. 4** Allele-specific Extended Haplotype Homozygosity (EHH) for four significant SNPs on
832 chromosomes 9.

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834 **Tables**

835 **Table 1.** Identification of significant SNPs and functional annotation of the plausible candidate
836 genes.
837

Significant SNP	Chromosome	Functional annotation of associated genes
ss715585948	Gm03	*WRKY* family transcription factor family protein Zinc fingers superfamily protein
ss715603454	Gm09	UDP-glucosyl transferase 88A1
ss715603455	Gm09	*RING/U-box* superfamily protein, *RING/FYVE/PHD* zinc finger superfamily protein
ss715603462	Gm09	*WRKY* family transcription factor family protein *MYB* domain
ss715603471	Gm09	Zinc fingers superfamily protein *Cytochrome P450* enzyme family Zinc finger, *RING-type*; Transcription factor jumonji/aspartyl beta-hydroxylase
ss715615975	Gm13	*bZIP* transcription factor *RING/U-box* superfamily protein, *RING/FYVE/PHD* zinc finger superfamily protein Zinc fingers superfamily protein *NAC* transcription factors *Cytochrome P450* enzyme family
ss715620269	Gm15	*RING/U-box* superfamily protein, *RING/FYVE/PHD* zinc finger superfamily protein *WRKY* family transcription factor family protein *MYB* domain

ss715636844	Gm20	UDP-Glycosyltransferase superfamily protein
		UDP-glucosyl transferase 85A2
		hydroxy methylglutaryl CoA reductase 1
		Cytochrome P450, family 71, subfamily B, polypeptide 34
		cytochrome p450 79a2
		RING/U-box superfamily protein, *RING/FYVE/PHD* zinc finger superfamily protein
		Zinc fingers superfamily protein

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839 **Table 2** Epistasis for the eight significant SNPs.

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	Ch9a	Ch9b	Ch9c	Ch9d	Ch13	Ch15	Ch20
Ch3	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.002*
Ch9a		0.10	0.053	0.007*	<0.001*	<0.001*	0.907
Ch9b			0.012	0.006*	<0.001*	<0.001*	0.835
Ch9c				<0.000*	<0.001*	<0.001*	n.e.
Ch9d					<0.001*	<0.001*	n.e.
Ch13						<0.001*	n.e.
Ch15							0.001*

841

842 Shown are the probabilities for each pairwise interaction of SNPs. * = $P < 0.05$ from
843 sequential Bonferroni tests. n.e. = not estimable. Ch3 = ss715585948, Ch9a = ss715603454, Ch9b
844 = ss715603455, Ch9c = ss715603462, Ch9d = ss715603471, Ch13 = ss715615975, Ch15 =
845 ss715620269, Ch20 = ss715636844

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851 **Figures**

852 **Fig. 1**

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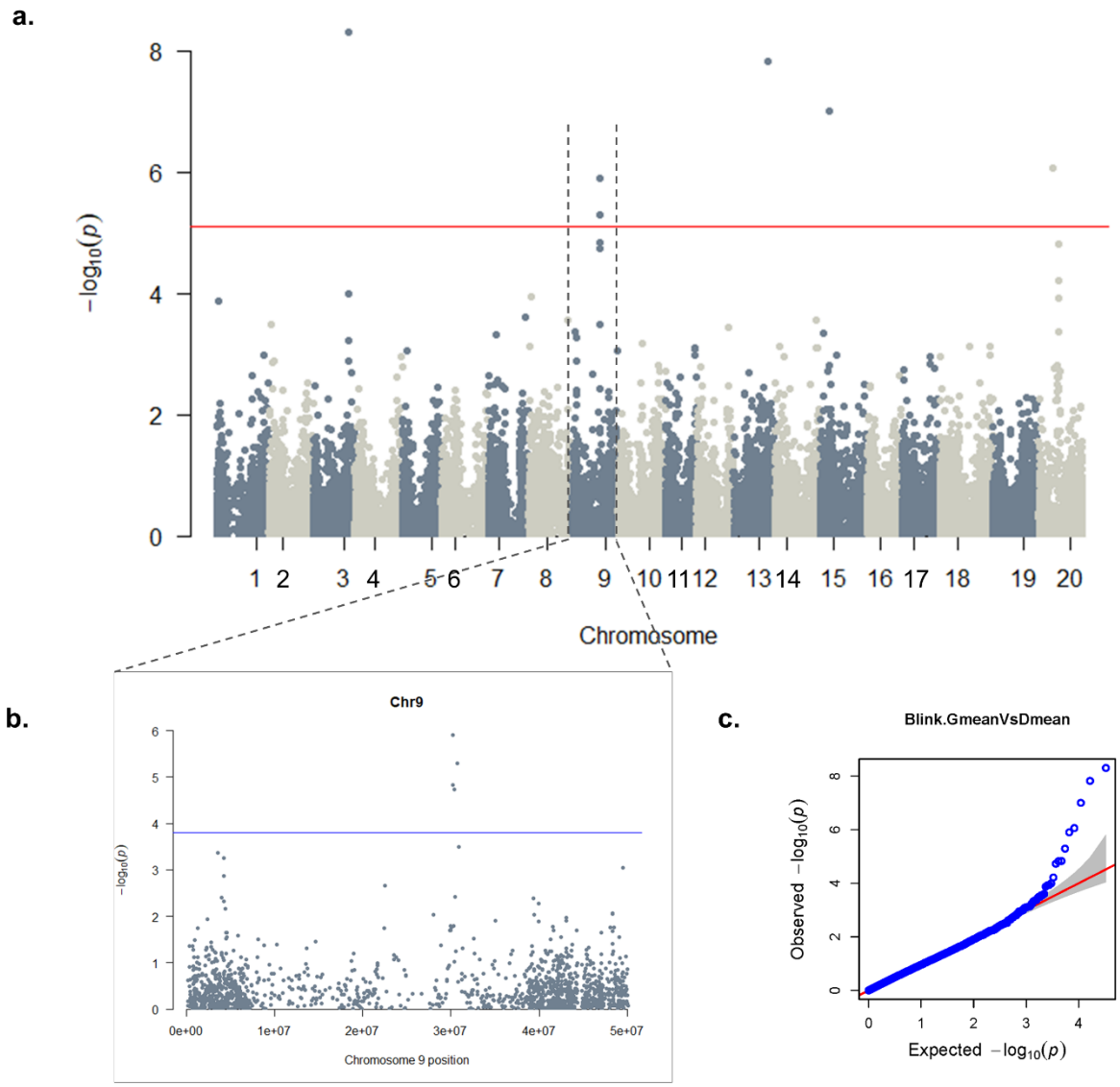

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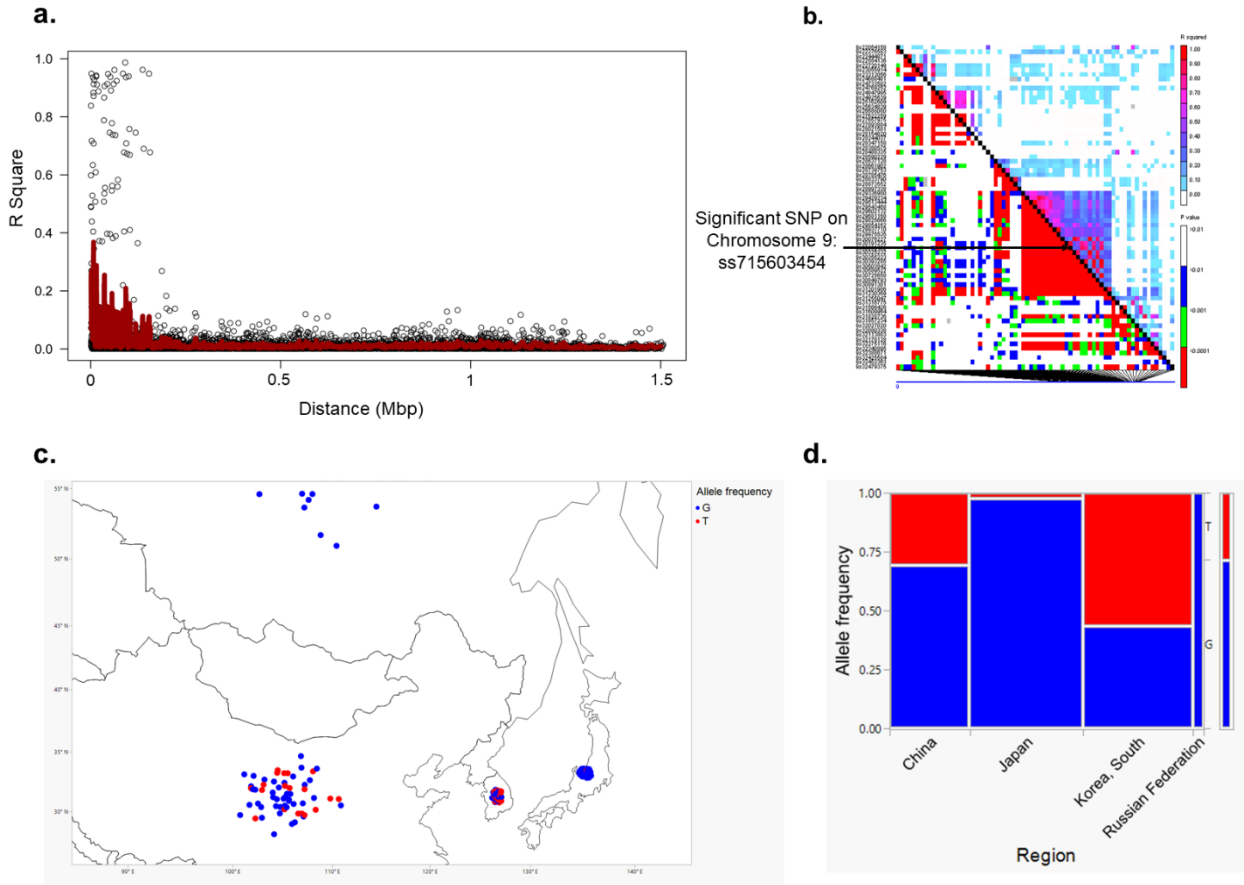
861 **Fig. 2**



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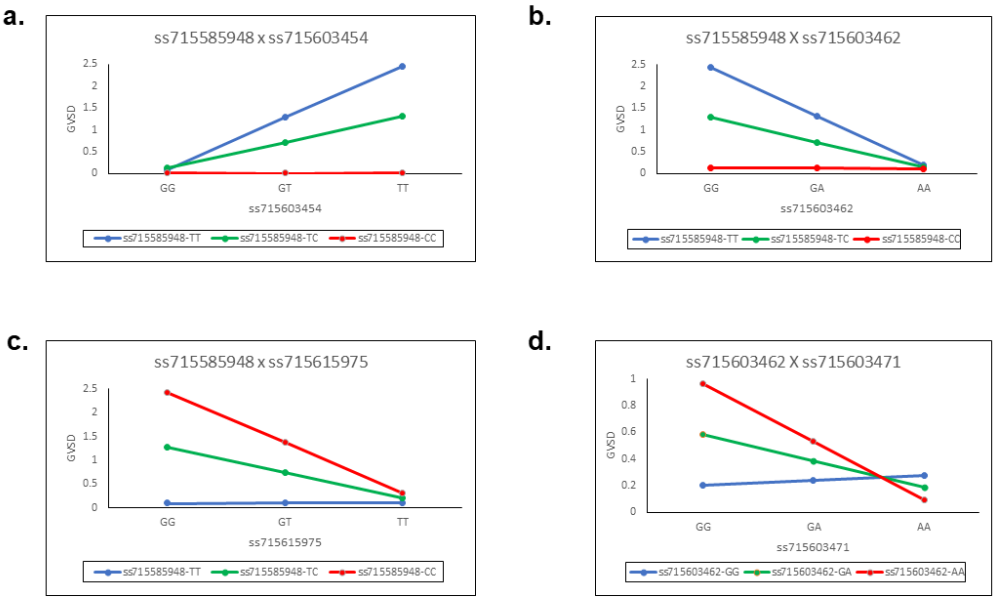
874 **Fig. 3**



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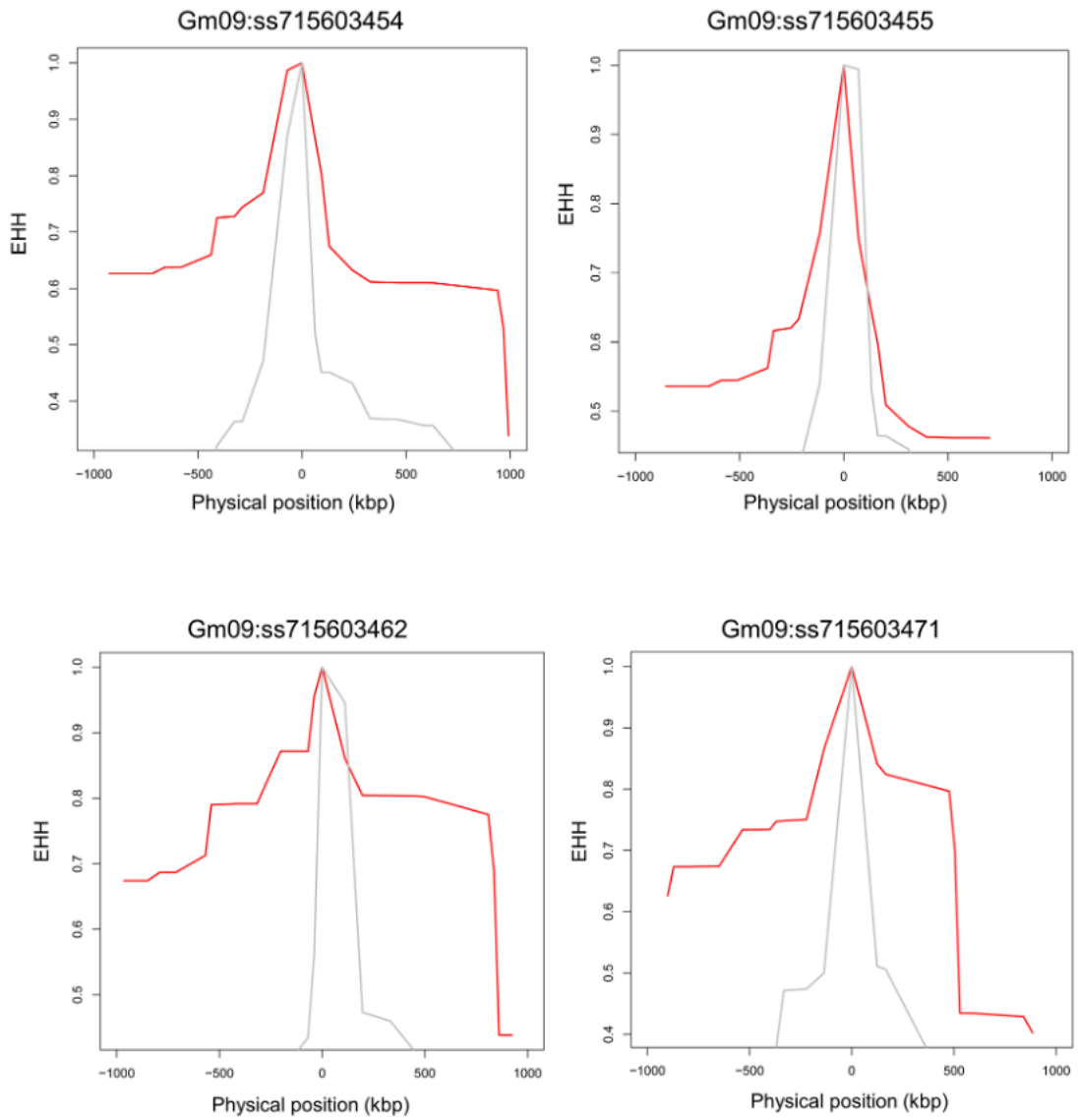
879 **Fig. 4**



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