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1 **Integrating omics approaches to discover and prioritize** 2 **candidate genes involved in oil biosynthesis in soybean**

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15

16 **Abstract**

17 Soybean is one of the major sources of edible protein and oil. Oil content is a quantitative
18 trait that is significantly determined by genetic and environmental factors. Over the past 30
19 years, a large volume of soybean genetic, genomic, and transcriptomic data have been
20 accumulated. Nevertheless, integrative analyses of such data remain scarce, in spite of their
21 importance for crop improvement. We hypothesized that the co-occurrence of genomic
22 regions for oil-related traits in different studies may reveal more stable regions encompassing
23 important genetic determinants of oil content and quality in soybean. We integrated publicly
24 available data, obtained with distinct techniques, to discover and prioritize candidate genes
25 involved in oil biosynthesis and regulation in soybean. We detected key fatty acid biosynthesis
26 genes (e.g., BCCP and ACCase, FADs, KAS family proteins) and several transcription factors,
27 which are likely regulators of oil biosynthesis. In addition, we identified new candidates for
28 seed oil accumulation and quality, such as Glyma.03G213300 and Glyma.19G160700, which
29 encode a translocator protein and a histone acetyltransferase, respectively. Further, oil and
30 protein genomic hotspots are strongly associated with breeding and not with domestication,
31 suggesting that soybean domestication prioritized other traits. The genes identified here are
32 promising targets for breeding programs and for the development of soybean lines with
33 increased oil content and quality.

34 **Keywords:** Seed oil content; fatty acids; differentially expressed genes; quantitative trait
35 loci; genome-wide association study.

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39 INTRODUCTION

40

41 Soybean (*Glycine max* (L.) Merr.) is a major source of protein and edible oil worldwide,
42 constituting a key factor in human and animal nutrition. With 17% to 22% seed oil content,
43 soybean is also widely used for industrial applications and biodiesel production (Abdelghany
44 et al., 2020). Soybean seed oil consists of triacylglycerol (TAG) ester molecules that
45 accumulate fatty acids (FAs) (Thelen & Ohlrogge, 2002), particularly palmitic (C16:0), stearic
46 (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. The proportion of these
47 FAs typically determines oil quality (Clemente & Cahoon, 2009). For example,
48 polyunsaturated fatty acids (PUFAs) are beneficial for human health (Sacks et al., 2017),
49 although the unsaturation degree and positions determine oil melting point (Voelker & Kinney,
50 2001). High PUFA levels, particularly that of linolenic acid, increase oil auto-oxidation and
51 reduce its useful life. Hence, a major goal in soybean genetic improvement is to increase oil
52 content and quality (e.g. increasing C18:1 content) (Clemente & Cahoon, 2009; Haun et al.,
53 2014; Liu et al., 2014), including the discovery of important genes involved in such phenotype
54 (Li et al., 2017; Liu et al., 2014; Zhang et al., 2019b; Zhang et al., 2016).

55 Oil biosynthesis involves different cell compartments and comprises a complex gene
56 network controlled by several quantitative trait loci (QTL) that are influenced by genetic and
57 environmental factors (Bates et al., 2013; Collard & Mackill, 2008; Schmidt & Herman, 2008).
58 In plants, *de novo* FA synthesis within plastids occurs through the coordination of several
59 metabolic pathways including the Calvin cycle, glycolysis, starch metabolism and the pentose
60 phosphate pathway (Bates et al., 2013; Gupta et al., 2017). TAGs are then assembled within
61 the endoplasmic reticulum and stored in oil bodies (Bates et al., 2013; Marchive et al., 2014).
62 Although well characterized in *Arabidopsis*, the genes involved in acyl-lipid metabolism are
63 not fully understood in soybean (Liu et al., 2020; Marchive et al., 2014). The difficulty to
64 functionally characterize these genes in soybean can be partially explained by the high
65 prevalence (i.e., ~75%) of protein-coding genes in multigene families, mainly because of two
66 whole-genome duplication events (Schmutz et al., 2010).

67 Over the past decades, several groups have explored the genomic complexity of oil-
68 related traits in soybean through linkage mapping (Akond et al., 2014; Bachlava et al., 2009;
69 Diers et al., 1992; Eskandari et al., 2013; Priolli et al., 2015; Qi et al., 2011; Vaughn et al.,
70 2014) and association mapping (i.e., Genome Wide Association Studies, GWAS) (Leamy et
71 al., 2017; Li et al., 2018; Zhang et al., 2018; Zhou et al., 2015). Detection of consensus QTL
72 have been used to define more stable QTL, i.e., those recurrently found across different
73 environments, often referred to as meta-QTL (MQTL) (Gong et al., 2018; Qin et al., 2018;
74 Van & McHale, 2017). Even though these studies have been extremely important in finding
75 key genes involved in agronomic traits, they often reveal long genomic segments comprising
76 many genes, requiring additional information to pinpoint causative genes or alleles. A rich
77 source of additional data can be found in transcriptomic studies, which have remarkably
78 accumulated over the past few years (Almeida-Silva et al., 2021; Bellieny-Rabelo et al., 2016;
79 Lu et al., 2016b). Recently, our group developed a comprehensive Soybean Expression Atlas
80 with 1,298 RNA-seq samples that can be used to investigate gene expression across different
81 tissues and conditions (Machado et al., 2020). This collection has also been used to build gene
82 co-expression networks (Almeida-Silva et al., 2020), which are instrumental in uncovering
83 important evolutionary trends among duplicated genes.

84 In spite of the large volume of association and linkage mapping, genomic, and
85 transcriptomic datasets, integrative approaches remain scarce (Liu et al., 2020; Niu et al.,
86 2020; Ronne et al., 2019), resulting in an incomplete picture of metabolic and regulatory
87 genes determining soybean oil content. Here, we integrate large-scale datasets from various
88 sources to define stable genomic regions and identify the most promising genes involved in
89 oil-related traits therein. The integrative strategy implemented here allowed the recovery of
90 genes known to be important for oil synthesis, as well as novel candidate genes to be
91 prioritized in experimental validation studies and in future crop improvement programs.

92 **RESULTS AND DISCUSSION**

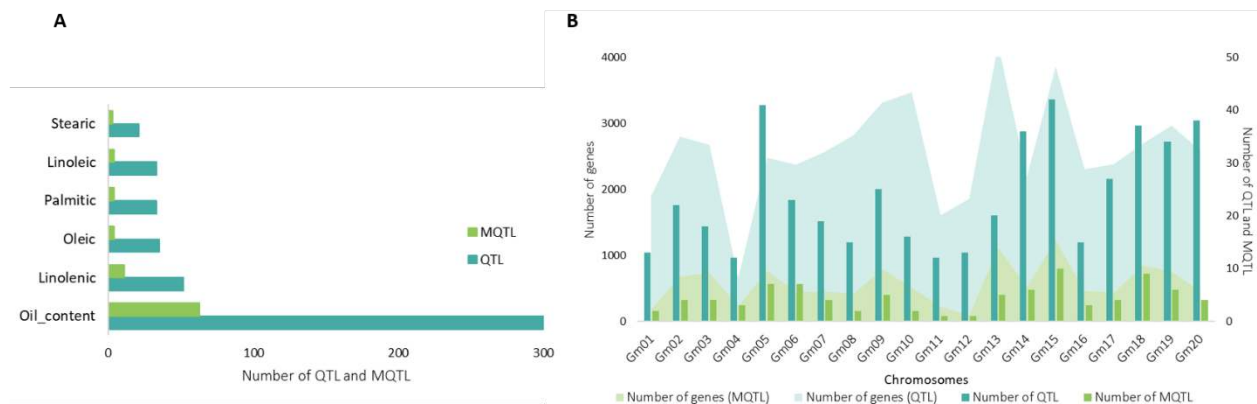
93 **Literature mining for QTL, MQTL, GWAS, selective sweep regions, and genes** 94 **associated with oil-related traits**

95 A total of 478 QTL controlling oil-related traits (Figure 1A) were retrieved from SoyBase
96 (soybase.org) (Supplementary Figure S1). We performed an initial exploratory analysis of the
97 distribution of these QTL along the soybean genome. The number of QTL per chromosome
98 ranged from 12 (in Gm04 and Gm22) to 42 (in Gm15), with an average of 23.9 QTL per
99 chromosome. The oil QTL sizes range from 0.004 Mb to 47.77 Mb, with an average length of
100 5.45 Mb. The largest oil QTL (47.77 Mb), comprising more than two thousand genes, was
101 greater than the average size (47.46 Mb) of the soybean chromosomes (Figure 1B;
102 Supplementary table S1).

103 Large confidence intervals are one of the main limitations of QTL analysis, as they
104 make the identification of causal genes a very challenging task (Borevitz & Nordborg, 2003;
105 Collard et al., 2008; Leamy et al., 2017). For example, the 478 QTL collected here encompass
106 40,268 genes, which correspond to 71.8% (40,268 / 56,044) of the soybean protein-coding
107 genes. Since many oil-related QTL are available, MQTL analyses can be used to better resolve
108 intervals and help identify effective candidate genes (Goffinet et al., 2000). Qi et al. found 89
109 MQTLs for oil-related traits (Qi et al., 2018). We found that 97.38% (11,104 / 11,403) of the
110 genes in MQTL intervals reported by Qi et al. are also in our QTL database (Figure 1A, B;
111 Supplementary table S1), which is a high correspondence given the several integrated
112 studies, methods, and different genome assembly versions used.

113 The progress in DNA sequencing technologies significantly improved the identification
114 of single nucleotide polymorphisms (SNPs) in the soybean genome (Song et al., 2013, 2016),
115 paving the way for GWAS (Daware et al., 2017; Mackay et al., 2009) and accelerating the
116 identification of genes with agronomic relevance. We searched the literature and retrieved
117 GWAS data from 15 publications, comprising a total of 458 SNPs significantly associated with
118 oil-related traits (Table 1; Supplementary figure S1). These SNPs allowed us to retrieve 344
119 statistically significant regions, encompassing 6,804 protein-coding genes (12.14% of the
120 soybean protein-coding genes) (Supplementary table S1 and S2).

121



122
123 **Figure 1.** Number of QTL and MQTL for each trait and their distribution on 20 chromosomes of soybean.
124 **A.** Number of QTL and MQTL for oil-related traits. **B.** Distribution of QTL and MQTL per chromosome
125 (bars) and their respective number of genes (shaded regions).
126
127

128 **Table 1.** Database of 15 Genome Wide Association Studies (GWAS) used in our analysis

Traits	SNPs	Origin	Accessions	Reference
O-P	23	China	279	(Li et al., 2019)
O-P	NA	China	104 RILs	(Karikari et al., 2019)
FA	149	China	194	(Zhao et al., 2019)
SC	25	Miscellaneous	313	(Zhang et al., 2018)
O	NA	USA	188 RILs	(Patil et al., 2018)
O-P	15	China	185	(Li et al., 2019)
O-P	11	China	421	(Li et al., 2018)
O-FA	NA	China	366	(Zhang et al., 2018)
O	4	China	279	(Cao et al., 2017)
O	25	Miscellaneous	809	(Fang et al., 2017)
O	79	USA	304	(Sonah et al., 2015)
O-P	18	USA	12,116	(Bandillo et al., 2015)
O-FA	51	Brazil	94	(Priolli et al., 2015)
FA	33	Miscellaneous	421	(Li et al., 2015)
O-P	25	USA	298	(Hwang et al., 2014)
Total	458			

129 **SNPs:** single nucleotide polymorphism (NA indicates that the number of SNPs was not informed); **RILs:** Recombinant
130 Inbred Lines; **O-P:** oil and protein; **O-FA:** oil and fatty acid; **O-P-FA:** oil, protein and fatty acid; **FA:** fatty acid; **SC:**
131 seed composition; **O:** oil.

132 Selective sweep is a process by which a strongly beneficial mutation spreads in a
133 population, increasing the frequency of linked neutral variants in a specific region and
134 dramatically reducing genetic variation in its vicinity (Chen et al., 2010; Stephan, 2019).
135 Allelic variation is lower in domesticated soybean accessions than in its wild relative *Glycine*

136 *soja*, most likely as a result of strong genetic bottlenecks, such as domestication and selective
137 breeding (Hyten et al., 2006; Liu et al., 2020). It is also clear that oil content was a major
138 target of artificial selection, resulting in increased oil content in cultivated soybean seeds (Wen
139 et al., 2015; Zhou et al., 2015). Hence, domestication and breeding can be used as a model
140 to uncover genes involved in recently selected traits (e.g. high seed oil content) through the
141 identification of selective sweep regions (Chen et al., 2010). Zhou et al. characterized several
142 selective signals related to domestication and breeding through genomic analyses of 302 wild,
143 landrace, and improved soybean accessions (Zhou et al., 2015). We retrieved a total of 2,230
144 genes within such selective sweep regions, which were categorized as selected during
145 domestication (59.24%), breeding (42.64%) or both (2.60%) (Supplementary table S1;
146 Supplementary figure S2).

147 We also gathered other datasets to enrich our analyses (Supplementary figure S2):
148 annotated transcription factors (TFs) (Moharana & Venancio, 2020; Niu et al., 2020; Yao et
149 al., 2020); hub genes from co-expression modules for oil-related traits (Qi et al., 2018; Yang
150 et al., 2019); differentially expressed genes (DEGs) between high and low-oil soybean
151 accessions (Niu et al., 2020) and DEGs for a critical period of oil accumulation during soybean
152 seed development (Yang et al., 2019). We leveraged these complementary datasets to better
153 understand the roles of these genes and prioritize candidates for crop improvement strategies,
154 as described below.

155

156 **RNA-Seq analyses and the identification of promising candidate genes for oil** 157 **accumulation and quality**

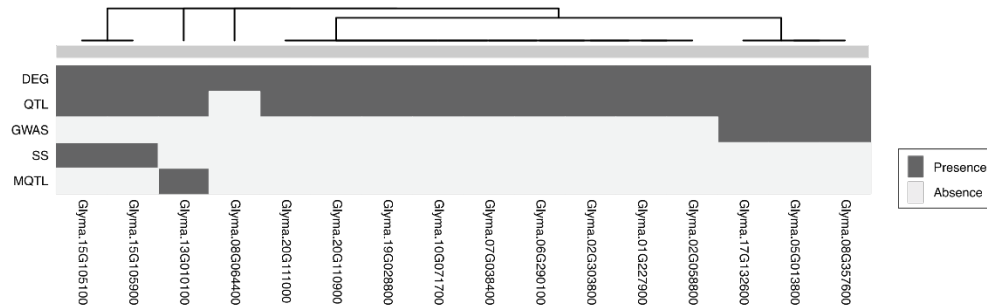
158 A recent study have identified 126 DEGs, during seed development of a single soybean line
159 with ~19% of oil content in a critical period of oil accumulation in seeds (Yang et al., 2019).
160 Another study reported 359 DEGs, from comparisons among six contrasting accessions for oil
161 content (11.9 to 12.5%; 17.2 to 17.8% and 20.9 to 22.3%) during seed development (Niu
162 et al., 2020). By comparing the DEGs from both studies, we found only 16 genes in common.
163 Glyma.01G227900 and Glyma.05G013800 encode steroleosin and oleosin, respectively;
164 Glyma.15G105900, Glyma.19G028800, Glyma.20G111000, and Glyma.13G010100 encode a
165 glucose-6-phosphate/phosphate translocator 2 (GPT2), a biotin carboxyl carrier protein
166 (BCCP), a fatty acid desaturase (FAD2-1B), and a long chain acyl-CoA synthesis (LACS8),
167 respectively; Glyma.15G105100 encode an aluminum-induced protein (AILP1);
168 Glyma.20G110900 (an ortholog of AT5G04750) encodes a mitochondrial F1F0-ATP synthase
169 inhibitor factor 1 that has been recently proposed to be crucial for plant growth and responses
170 to abscisic acid (ABA) in *A. thaliana* (Chen et al., 2020); Glyma.08G064400 encodes a protein
171 of unknown function. We also found TFs from the following families: bZIP (Glyma.02G058800
172 and Glyma.10G071700), NF-YA (Glyma.02G303800), C3H (Glyma.06G290100), GRF
173 (Glyma.07G038400), B3 (Glyma.08G357600), and TALE (Glyma.17G132600). Interestingly,
174 15 of the 16 genes listed above are located in QTL, GWAS or selective sweep regions (Figure
175 2). Unexpectedly, only Glyma.13G010100 was found in MQTL regions. This result not only
176 enforces the need for integrating different sources of genomic information, but also indicates
177 that MQTL cannot directly displace QTL data in integrative analyses. Notably, 50%, 43.75%
178 and 6.25% of these 16 genes encode oil biosynthesis or storage genes, TFs and genes of
179 unknown function, respectively (Figure 2; Supplementary table S3). This remarkable

180 representation support TFs as key regulators of transcriptional programs involved in oil
181 accumulation during seed development. Originally, among the DEGs reported by Yang et al.
182 and Niu et al., 9.68% and 29.33% are TFs, respectively (Niu et al., 2020; Yang et al., 2019).
183 This trend has prompted us to further investigate TFs related with oil content in soybean,
184 which are discussed in the next section.

185 Interestingly, 6 of the 16 genes reported above (i.e. GPT2, BCCP, FAD2-1B, LACS8,
186 steroleosin and oleosin) are well described as involved in oil biosynthesis and storage (Haun
187 et al., 2014; Li et al., 2016; Salie et al., 2016a). GPT2 encodes a plastid transporter that
188 imports glucose-6-phosphate into plastids, fueling FA synthesis. GPT2 was likely selected
189 during soybean domestication (Figure 2). GPT2 is 9 times more expressed in oil palm (*Elaeis*
190 *guineensis*), which accumulates up to 90% oil in its mesocarp, than in date palm (*Phoenix*
191 *dactylifera*), which stores almost exclusively carbohydrates (Bourgis et al., 2011). BCCP
192 encodes a subunit required for ACCase activity, which catalyzes the committed step of de
193 novo FA synthesis (Salie & Thelen, 2016b). FAD2 encodes a FA desaturase that catalyzes the
194 formation of C18:2 from C18:1 (Haun et al., 2014), while LACS8 is involved in FA export from
195 plastid to TAG synthesis (Li et al., 2016). It has been proposed that the upregulation of LTPs
196 (lipid transporters) and LACs could improve the efficiency of lipid transport and increase oil
197 content (Koo et al., 2004; Manan et al., 2017; Niu et al., 2020). During seed development,
198 there is the deposition of oil bodies, which are TAGs surrounded by a monolayer membrane
199 containing steroleosin and oleosin, among other proteins (Lin et al., 2002; Schmidt et al.,
200 2008). Interestingly, Glyma.20G110900 (related to aerobic respiration) and
201 Glyma.08G064400 (unknown function) have been previously associated to oleic acid content
202 in soybean (Liu et al., 2020; Niu et al., 2020).

203 We found that Glyma.03G213300 (an ortholog of AT2G47770), which encodes a
204 membrane translocator protein (TSPO), was upregulated in a critical period of oil accumulation
205 in seeds (Yang et al., 2019). Located at the membrane of the endoplasmic reticulum and Golgi
206 complex (Guillaumot et al., 2009), TSPO is expressed in *Arabidopsis* seeds and induced by
207 osmotic treatment, salt stress and ABA (Guillaumot et al., 2009). Further, TSPO is involved
208 in energy homeostasis by promoting the accumulation of FAs and oil bodies in mature seeds
209 (Jurkiewicz et al., 2018). Accordingly, we found that TSPO expression is greater in soybean
210 seed tissues (Supplementary figure S3), leading us to hypothesize it as a strong candidate
211 for oil biosynthesis to be prioritized in experimental validation.

212



213

214 **Figure 2.** Integration of the genes found by Yang et al. and Niu et al. (Niu et al., 2020; Yang et al.,
215 2019). The heatmap shows the presence/absence of these genes in genomic regions for oil-related
216 traits. DEG, differentially expressed gene. QTL, quantitative trait loci. GWAS, genome wide association
217 studies. SS, selective sweep regions. MQTL, meta-QTL.

218

219 These results corroborate the importance of integrating different datasets to find
220 relevant candidate genes, since genes known to act in oil biosynthesis are often not detected
221 by all methods because of technical limitations (Korte & Farlow, 2013; Mackay et al., 2009)
222 or biological contexts. For example, several classic genes involved in oil biosynthesis were not
223 found in GWAS or MQTL regions (e.g. Glyma.19G028800 and Glyma.20G111000). On the
224 other hand, the integration of transcriptomic data can help discriminating interesting
225 candidates from large QTL intervals (Figure 2; Supplementary table S3).

226

227 **Transcription factors in genomic regions for oil-related traits**

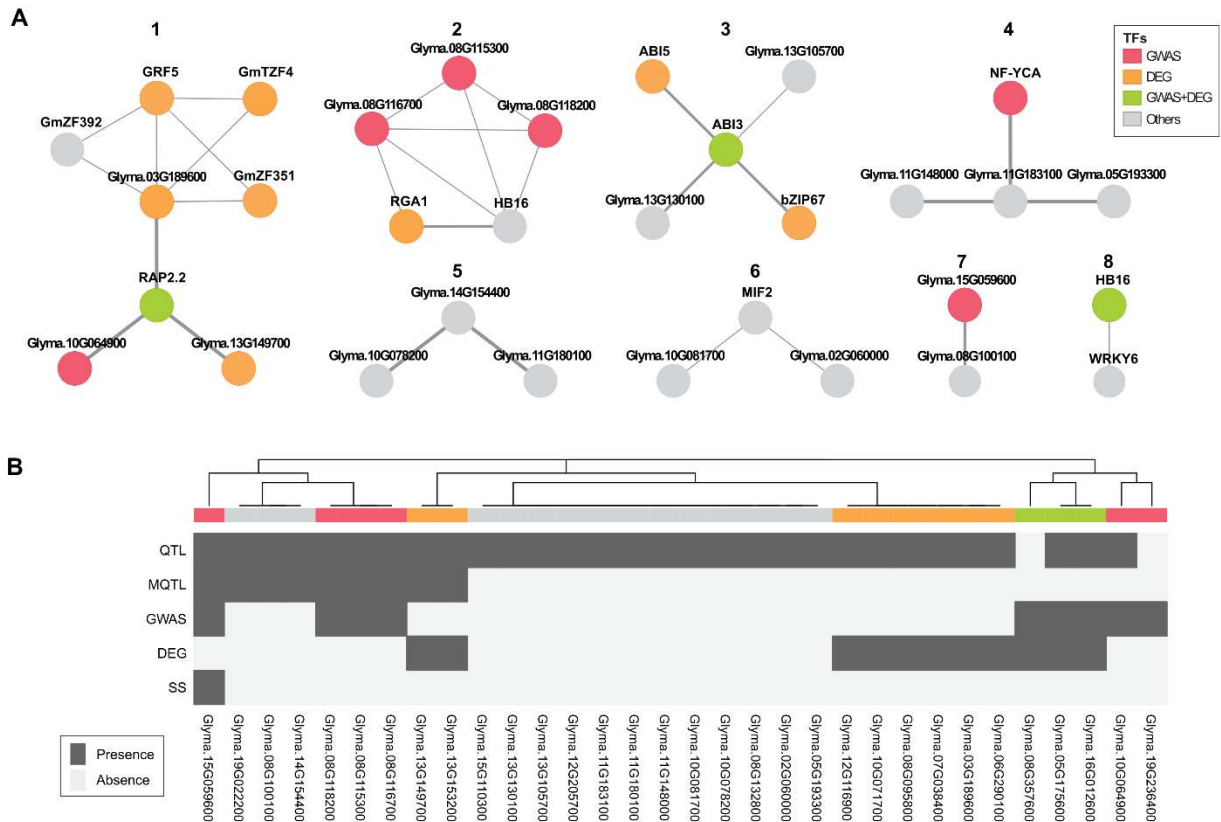
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229 TFs integrate various signals that coordinate metabolic pathways, including oil biosynthesis
230 (Manan et al., 2017). Despite the knowledge about many TFs involved in oil biosynthesis
231 (Kanai et al., 2019; Pham et al., 2012; Sandhu et al., 2007), several regulators and their
232 regulatory interactions remain unknown (Kong et al., 2020; Wang & Komatsu, 2017). To this
233 end, we investigated the occurrence of soybean TFs in oil-related regions, i.e., QTL, MQTL,
234 GWAS or selective sweep regions. Out of the 3,450 unique TFs considered here, 77.53% were
235 within genomic regions for oil-related traits (Supplementary table S4; Supplementary figure
236 S2). Because of the reasons outlined above, this number is obviously overestimated.

237 GmZF351 (Glyma.06G290100) increases oil content in soybean seeds by promoting
238 the expression of the WRI1 TFs (Glyma.15G221600; Glyma.08G227700) and other lipid
239 biosynthesis genes, namely BCCP2 (Glyma.19G028800), KASIII (Glyma.15G003100), TAG1
240 (Glyma.17G053300; Glyma.13G106100) and OLEO2 (Glyma.16G071800;
241 Glyma.19G063400) (Li et al., 2017). GmZF392 (Glyma.12G205700), a homolog of GmZF351,
242 is also important for lipid accumulation. GmZF392 and GmZF351, which are 51.3% identical,
243 regulate distinct genes and physically interact with each other to activate downstream genes
244 (Lu et al., 2021). Motivated by cases like this, we selected a total of 284 TFs within oil-related
245 regions. We used TFs found in at least two of the three studies mentioned earlier (Moharana
246 & Venancio, 2020; Niu et al., 2020; Yao et al., 2020) (Supplementary table S5) to investigate
247 the TF interaction patterns in the STRING database (Szklarczyk et al., 2017).

248 From the 284 selected TFs, 31 had interactions in STRING, of which 35.48%, 29.03%
249 and 9.68% were up-regulated in high oil content accessions of soybean, detected in GWAS or
250 both, respectively (Supplementary table S5). GmZF392 was found in only one of the three TF
251 datasets explored here, but was kept in the analysis based on the strong experimental
252 evidence supporting its role in oil biosynthesis. Therefore, Figure 3 shows 32 TFs clustered in
253 eight groups. Groups 1,2,3, and 8 showed genes upregulated in high oil content accessions
254 and will be discussed below.

255 Group 1 contains eight TFs from the families: GRF (Glyma.07G038400 [GRF5]), ERF
256 (Glyma.16G012600 [RAP2.2]), Trihelix (Glyma.03G189600; Glyma.10G064900;
257 Glyma.13G149700), and C3H (Glyma.06G290100 [GmZF351]; Glyma.12G116900
258 [GmTZF4]; Glyma.12G205700 [GmZF392]). Interestingly, GmZF351 and GmZF392 showed
259 the same interactions in the network (Figure 3A). From the eight genes in Group 1, six were
260 upregulated in high oil content accessions (Figure 3B). GRF5, Glyma.03G189600 and GmTZF4
261 were previously predicted to be candidates in regulation of seed lipid biosynthesis (Niu et al.,
262 2020; Niu et al., 2020; Zhang et al., 2016). Group 2 shows associations among five TFs from
263 the families GRAS (Glyma.08G095800 [RGA1]), HD-ZIP (Glyma.08G132800 [HB16]), bZIP
264 (Glyma.08G115300), G2-like (Glyma.08G116700), and WRKY (Glyma.08G118200). The
265 relationship between these TFs in the regulation of oil biosynthesis unclear, although mutants
266 of RGA1 – a negative regulator in the gibberellin signaling pathway – have alterations in seed
267 fatty acid metabolism in *Arabidopsis* (Chen et al., 2012). Group 3 shows associations among
268 five TFs from the family B3 (Glyma.08G357600 [ABI3]), bZIP (Glyma.10G071700 [ABI5];
269 Glyma.13G153200 [bZIP67]), HSF (Glyma.13G105700), and bHLH (Glyma.13G130100).
270 Among them, ABI3, ABI5, and bZIP67 were found to be involved in oil biosynthesis (Mendes
271 et al., 2013; Zhang et al., 2016; Zhang et al., 2017). Group 8 shows the association between
272 Glyma.05G175600 (HD-ZIP family, HB16) and Glyma.15G110300 (WRKY family, WRKY6).
273 WRKY6 was downregulated in high oil content accessions (Niu et al., 2020). We believe that
274 interaction maps like the one presented here, integrating various sources of evidence, can
275 help us understand the regulatory dynamics involved in oil biosynthesis and in the discovery
276 of new potential regulators.



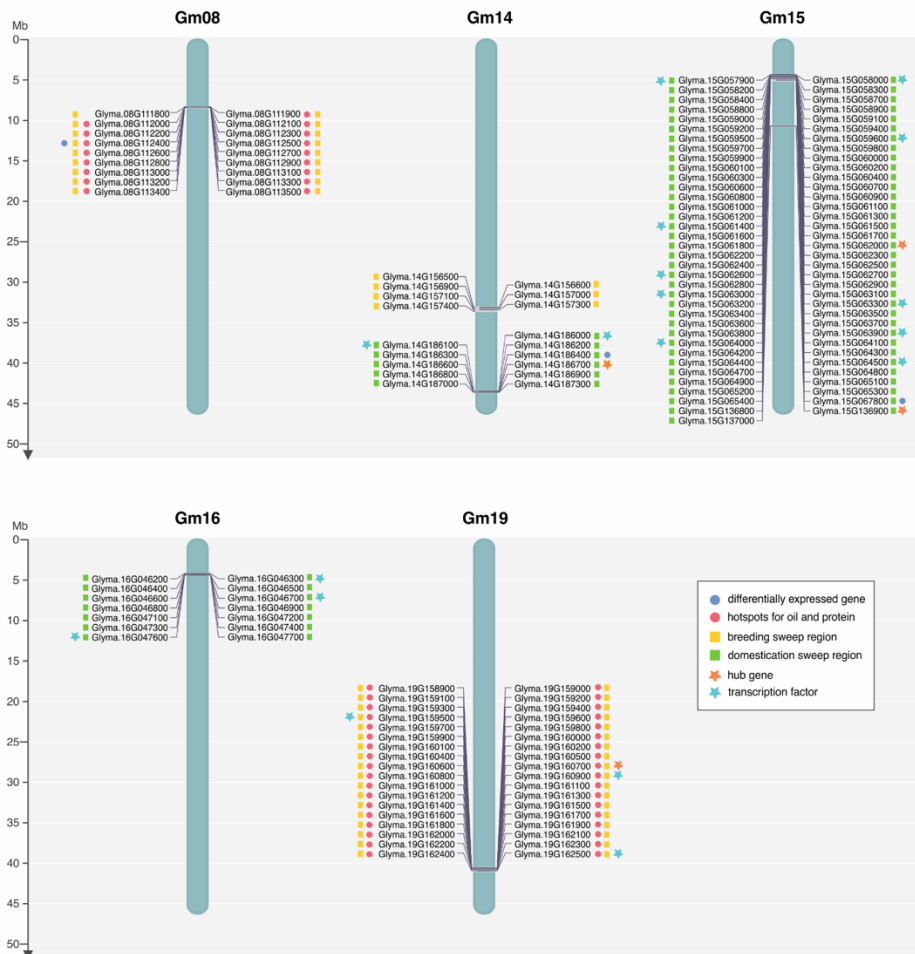
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279 **Figure 3.** Interaction analysis candidate transcription factors (TFs). **A.** The TFs in eight interaction
 280 groups constructed using the STRING database (Szklarczyk et al., 2017). These associations refer to
 281 physical or functional relationships with a high confidence interaction score (0.7), where the edge weight
 282 is proportional to the support (Supplementary table S6). Nodes were colored as follows: red (genes
 283 present GWAS regions); orange (differentially expressed gene – DEG – upregulated when comparing
 284 high and low oil content soybean accessions); green (genes reported in both GWAS and DEG); gray
 285 (genes previously reported in quantitative trait loci [QTL], meta-QTL [MQTL] or selective sweep regions
 286 [SS]). **B.** Presence/absence profiles of the 32 candidate TFs in genomic regions for oil-related traits. The
 287 bar above the heatmap was colored as described in panel A.

288 Identification of candidate genes for oil-related traits

289 Since the first QTL study of oil-related traits in soybean (Diers et al., 1992), a large set of
 290 genomic data have accumulated, including hundreds QTL and GWAS regions, as well as loci
 291 that underwent positive selection during domestication and/or breeding (Zhou et al., 2015),
 292 as discussed above. As demonstrated in this work, integrating such data can help mitigate
 293 the individual limitations of each technique. For example, QTL mapping is efficient in finding
 294 rare genes of large effect in artificial populations, but only alleles that segregate between the
 295 F2 and its progeny can be assessed (Mackay et al., 2009). On the other hand, GWAS work
 296 well with natural populations, but is limited in detecting rare alleles in a population (Korte &
 297 Farlow, 2013). Furthermore, identifying domestication or breeding selective sweep regions
 298 may also unveil powerful candidate genes (Han et al., 2016). We integrated the positional

299 information from MQTL, GWAS and selective sweep regions to better understand the context
 300 of oil-related genes therein. These datasets contain 11,403, 6,229, and 2,230 genes,
 301 respectively (Supplementary table S1; Supplementary figure S2). In total, we found 157
 302 genes co-located in MQTL, GWAS, and selective sweep regions (Supplementary table S7;
 303 Supplementary figure S4). Remarkably, 33.75% (53/157) of these genes are within hotspot
 304 regions for oil and protein (Qi et al., 2018), i.e. those reported in at least four different
 305 soybean QTL studies related with both, oil and protein content. The 157 genes are located in
 306 well defined regions of Gm08 (8.61 – 8.71 Mb), Gm14 (34.25 – 34.74 Mb and 45.0 – 45.2
 307 Mb), Gm15 (4.47 – 5.17 Mb and 11.06 – 11.12 Mb), Gm16 (4.36 – 4.53 Mb), and Gm19
 308 (41.94 – 42.32 Mb) (Figure 4). Importantly, these hotspot regions comprise only 1.949 genes
 309 (Qi et al., 2018).



310
 311 **Figure 4.** Chromosome map with 157 genes co-located in MQTL, GWAS, and selective sweep regions.
 312 Differentially expressed genes were obtained by a comparison of high and low oil content soybean
 313 accessions (Niu et al., 2020). Hotspots for oil and protein and selective sweep regions were reported by
 314 Qi et al. (2018) and Zhou et al. (2015), respectively. Hub genes are those with the highest number of
 315 interactions in expression modules positively correlated with oil-related traits (Qi et al., 2018; Yang et
 316 al., 2019). More details on the annotations of these genes are provided in Supplementary table S7.
 317

318 Some remarkable trends emerge from these regions. The Gm08 and Gm19 regions
319 harbor breeding sweep regions and are associated with oil and protein content. Similarly, all
320 the Gm15 and Gm16 blocks corresponded only to domestication sweep regions. We also found
321 that oil and protein hotspots are strongly associated with breeding and never with
322 domestication, suggesting that domestication prioritized the selection of other traits.
323 Interestingly, 15 of 18 TFs found among the 157 genes within these blocks are associated
324 with domestication. Together, these results indicate that domestication involved the selection
325 of regulatory programs, whereas breeding appears to involve mainly lipid and other metabolic
326 genes (Supplementary table S8). It is also conceivable that several TFs selected during
327 domestication regulate transcriptional programs that were important for breeding.

328 We further investigated the functions of the 157 genes within these regions using gene
329 coexpression networks from two studies, in particular with regard to the hubs from oil-related
330 modules. Qi et al. found 96 hub genes in a module (brown) positively correlated with oil-
331 related traits (Qi et al., 2018). Of these, four (i.e. Glyma.14G186700, Glyma.15G062000,
332 Glyma.19G160700, and Glyma.15G136900) are among the 157 genes reported above (Figure
333 4; Supplementary table S9; Supplementary figure S5). These genes are likely involved in
334 defense/immunity (Glyma.14G186700; Glyma.15G062000) or regulatory processes
335 (Glyma.19G160700; Glyma.15G136900). The only hub gene in a breeding region is
336 Glyma.19G160700 (an ortholog of AT3G54610), which encode a GNAT histone acetyl
337 transferase (GCN5). Wang et al. revealed that GCN5-dependent H3K9/14 acetylation of
338 Omega-3 fatty acid desaturase (FAD3) determined the expression levels of FAD3 in *A. thaliana*
339 seeds. Moreover, the ratio of linolenic to linoleic acid in the *gcn5* mutant was rescued to the
340 wild-type levels through the overexpression of FAD3 (Wang et al., 2016). These results make
341 Glyma.19G160700 a promising candidate to improve soybean oil quality.

342 Yang et al. found a total of 31 hubs in modules (pink, brown and blue) positively
343 correlated with oil-related traits (Yang et al., 2019). Interestingly, five (16.13%) of these
344 hubs (Glyma.03G204400, Glyma.05G234000, Glyma.06G195000, Glyma.07G196200, and
345 Glyma.19G228800) are reported in at least two different studies considered here
346 (Supplementary table S9; Supplementary figure S5). Among them, Glyma.05G234000 (an
347 ortholog of AT5G16110) encodes a hypothetical protein. According to data from TAIR, although
348 this gene is expressed in several tissues and its protein product locates to the chloroplast,
349 there is no evidence about its molecular functions or conserved domains (Rhee et al., 2003).
350 Glyma.19G228800 (an ortholog of AT4G02080) encodes an ADP-ribosylation factor-relate
351 (ARF1) that can be involved in transport from the endoplasmic reticulum to the Golgi
352 apparatus (Matheson et al., 2007). Glyma.03G204400 (an ortholog of AT5G22000) present a
353 RING-H2 conserved domain. Previous studies have shown that RING-finger proteins are
354 involved in plant growth and development, stress resistance, hormone signaling responses
355 and controlling characteristics of both vegetative and seed yield (Sun et al., 2019; Zombori
356 et al., 2020). Glyma.06G195000 (an ortholog of AT2G03090) present two expansin domains,
357 a pollen allergen domain, and a rare lipoprotein A (RlpA)-like domain. Finally,
358 Glyma.07G196200 (an ortholog of AT4G21610) encode a LSD1 zinc finger, involved in rice
359 growth and disease resistance (Xu & He, 2007). The roles of these genes in oil biosynthesis
360 are yet to be characterized.

361 The negative correlation between oil and protein contents in soybean has been widely
362 reported over decades (Bandillo et al., 2015; Chaudhary et al., 2015; Johnson & Bernard,

363 1962; Patil et al., 2017). Hence, identifying regions that simultaneously contribute to these
364 traits have been a topic of great interest. In hotspot regions, we found Glyma.08G112300 (an
365 ortholog of AT1G55260) that encodes a multifunctional 2S albumin superfamily protein
366 involved in defense and storage (Lin et al., 2004). Glyma.08G112300 has been reported as a
367 strong determinant of high levels of water-soluble proteins, a critical factor both in food quality
368 and in the production of isolated soybean proteins (Zhang et al., 2017). However, this gene
369 is highly expressed across several tissues (Supplementary figure S6). Curiously, only 2kb
370 away from Glyma.08G112300, we found Glyma.08G112400 (Figure 4; Supplementary figure
371 S7; Supplementary table S7), which encodes a protein with a domain of unknown function
372 DUF538 that has been recently proposed as a candidate gene to determine oleic acid content
373 (Niu et al., 2020). The genomic closeness of these two genes indicate that they should be
374 investigated in more detail with regard to their roles in determining protein and oil content.

375 **CONCLUSIONS**

376 Here, we used integrative approaches to explore genes in stable genomic regions for oil-
377 related traits in soybean. We explored publicly available datasets, mainly from studies of QTL,
378 MQTL, GWAS, and selective sweep regions. This core dataset was complemented with gene
379 expression data, gene expression networks and TF annotations to help elucidating the genetic
380 basis of oil-related traits. The integrative analyses reported here provide a framework to
381 identify and prioritize candidate genes. Finally, the gene set reported here might be an
382 important repository for experimental validation and soybean improvement programs.

383 **MATERIALS AND METHODS**

384 **Oil-related QTL and GWAS data**

385 Coordinates of QTL and several genetic markers were retrieved from SoyBase (update from
386 January 2018; soybase.org). QTL were extracted with in-house Perl and bash scripts. The
387 flanking regions of the closest genetic markers were used to define the ends of each QTL.
388 Next, we used a bash script to extract a total of 4,352 names of QTL objects, which had their
389 chromosomal coordinates determined. From these, we found 478 oil-related QTL
390 (Supplementary table S10) and retrieved the genes therein using the file
391 Gmax_275_Wm82.a2.v1.gene.gff3, from Phytozome V12.1, as reference. The files containing
392 the coordinates of 478 oil-related QTL and genes (gff3) were integrated in Browser Extensible
393 Data (BED) format with Bedtools V2.27.1 (Quinlan, 2014), followed by redundancy removal
394 (Supplementary figure S1; Table S1). The GWAS data were obtained from 15 studies (Table
395 1). The coordinates of the significant LD regions (Supplementary table S2), corresponding to
396 the SNPs reported in the original studies, were collected in individual files. Six of these studies
397 used an older version of the soybean reference genome (Glyma.Wm82.a1.v1) and had their
398 data converted using the same reference genome file mentioned above. These files were
399 combined and processed to remove redundancy, resulting in a GWAS list with 6,804 genes
400 (Supplementary figure S1; Supplementary table S1).

401 **Lipid metabolism pathways**

402 Information about genes in metabolic pathways were obtained from soybase.org and from
403 Yao et al. 2020 (Supplementary table S8 (column QTL-LM); Supplementary table S11).

404 **MQTL, hub genes, DEGs, selective sweep and transcription factors**

405 We supplemented our data using MQTL, hub genes from modules positively correlated with
406 oil, DEGs from soybean accessions of low to high oil content, positively selected (selective
407 sweep) regions during seed domestication/breeding and TF classifications. The chromosomal
408 map with genes co-located in selective sweeps, MQTL, and GWAS regions was constructed
409 using MapGene2Chrom (Jiangtao et al., 2015). A workflow is available at Supplementary
410 figure S2.

411 The coordinates of MQTL were obtained from Qi et al. (Qi et al., 2018). We retrieved
412 63, 26 and 11 MQTL for oil content, fatty acid and hotspots for oil and protein, respectively
413 (Supplementary tables S12 and S13). MQTL coordinates and genes were retrieved with the
414 same strategy used for QTL. We also used hubs from a coexpression modules reported by
415 two previous studies (Supplementary table S8) (Qi et al., 2018; Yang et al., 2019).

416 We used DEGs from two publications. Yang et al. reported DEGs between 20 and 10
417 days after flowering – a critical seed oil accumulation stage in the soybean variety
418 ‘nannong1138-2’ (NN1138-2), which shows ~19% seed oil content (Yang et al., 2019). The
419 second dataset, from Niu et al. encompasses six soybean accessions with ~11% to 22% seed
420 oil content (Niu et al., 2020). Selective sweep regions (Zhou et al., 2015) were retrieved and
421 had their gene names/coordinates updated as described above for the GWAS (Supplementary
422 table S8).

423 TFs were recovered from three sources. TFs reported in stable QTL regions (Yao et al.,
424 2020); TFs differentially expressed in soybean accessions with divergent oil content and
425 compositions (Niu et al., 2020); and from a systematic classification of legume TF repertoires
426 (Moharana & Venancio, 2020). These data are available in Supplementary table S8. TF
427 interaction analysis was conducted using STRING version 11 (Szklarczyk et al., 2017), using
428 a 0.7 (high) confidence threshold. To the input we used the file
429 Gmax_275_Wm82.a2.v1.protein.fa. from Phytozome V12.1, as reference.

430 **Global gene expression**

431 Analyses of global expression genes were conducted from Soybean Expression Atlas database
432 (Machado et al., 2020), using Kallisto as a method for estimating gene expression.

433

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441

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