DELLA proteins BnaA6.RGA and BnaC7.RGA negatively regulate fatty acid biosynthesis by interacting with BnaLEC1s in Brassica napus

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Summary
Seed oil content (SOC) and fatty acid (FA) composition determine the quality and economic value of rapeseed (Brassica napus). Little is known about the role of gibberellic acid (GA) in regulating FA biosynthesis in B. napus. Here, we discovered that four BnaRGAs (B. napus REPRESSOR OF GA), encoding negative regulators of GA signalling, were suppressed during seed development. Compared to the wild type, SOC was reduced in gain-of-function mutants bnaa6.rga-D and ds-3, which also showed reduced oleic acid and increased linoleic acid contents. By contrast, the loss-of-function quadruple mutant bnarga displayed higher SOC during early seed development than the wild type, with increased oleic acid and reduced linoleic acid contents. Notably, only BnaA6.RGA and BnaC7.RGA physically interacted with two BnaLEC1s, which function as essential transcription factors in FA biosynthesis. The FA composition did not significantly differ between bnaa6.rga-1 sextuple mutants and bnaa6.rga-1, suggesting that BnaLEC1s are epistatic to BnaRGAs in the regulation of FA composition. Furthermore, BnaLEC1-induced activation of BnaAB13 expression was repressed by BnaA6.RGA, indicating that GA triggers the degradation of BnaRGAs to relieve their repression of BnaLEC1s, thus promoting the transcription of downstream genes to facilitate oil biosynthesis. Therefore, we uncovered a developmental stage-specific role of GA in regulating oil biosynthesis via the GA-BnaRGA-BnaLEC1 signalling cascade, providing a novel mechanistic understanding of how phytohormones regulate FA biosynthesis in seeds. BnaRGAs represent promising targets for oil crop improvement.

Keywords: Brassica napus, GA, BnaRGAs, FAs, BnaLEC1s.

Introduction
Brassica napus (AACC, 2n = 38) is a major oil crop, providing approximately 16% of oils globally (Woodfield et al., 2017). The high levels of unsaturated fatty acids (FAs; >60%) and the low level of erucic acid (C22:1; <1%) in canola oil have made it widely accepted as a vegetable oil for human consumption and as a biofuel for industry (Dupont et al., 1989; Lin et al., 2013). In recent years, the demand for vegetable oil has sharply increased. Therefore, increasing the oil content in B. napus seeds is a major goal for the genetic improvement of this crop.

Triacylglycerol (TAG), which is mainly stored in seeds, is synthesized from glycerol-3-P and FAs (Yang and Benning, 2018). In Arabidopsis thaliana, transcription factors encoding genes, such as LEC1 (LEAFY COTYLEDON1), LEC2, ABI3 (ABSCISIC ACID-INSENSITIVE3), FUS3 (FUSCA3) and WRI1 (WRINKLED1), play important roles in regulating seed oil accumulation (Santos-Mendoza et al., 2008; To et al., 2006). Loss-of-function mutations of these genes usually lead to severe defects in oil storage (Braybrook and Harada, 2008; Meinke et al., 1994). Conversely, ectopic expression of these activators confers TAG accumulation in transgenic rosette leaves (Gao et al., 2009; Tsukagoshi et al., 2006). For example, more than 50% of the genes involved in FA biosynthesis in Arabidopsis plastids are regulated by LEC1; the relative mRNA levels of these genes are increased within a few days of flowering.

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hours following chemical activation of an inducible LEC1 transgene (Jo et al., 2019; Mu et al., 2008). Besides regulating FA biosynthesis, LEC1 also influences the FA composition of seeds. Seed-specific ectopic expression of AtLEC1 in peanut results in increased oleic acid (C18:1) and decreased of linoleic acid (C18:2) contents (Tang et al., 2018). However, overexpressing one of the \( B. \) \( napus \) LEC1 genes \((BnaLEC1)\) under the control of the napA promoter in \( B. \) \( napus \) leads to higher levels of C18:2 and lower levels of C18:1 (Tan et al., 2011). Because FA composition affects oil quality, it is important to further clarify the roles of LEC1 in FA biosynthesis.

A few studies have investigated the effects of phytohormones on seed oil content (SOC). Treatment with gibberellins (GAs) increases the levels of unsaturation of FAs in the aleurone layer of barley seeds (Villasuso et al., 2013). Exogenous GA treatment of \( B. \) \( napus \) during seed development increases the seed oil content (Huang et al., 2014). By contrast, GA negatively regulates lipid accumulation in Arabidopsis seeds by up-regulating a group of GDSL-type lipase genes (Cao et al., 2006; Chen et al., 2012), suggesting that GA plays dual roles in controlling FA accumulation.

DELLA proteins are conserved growth repressors that modulate all aspects of GA responses (Nakahima et al., 2006). DELLA proteins regulate vegetative growth and fruit development by interacting with DELLA-interacting proteins (DIPs) (Davière and Achard, 2016). \( B. \) \( napus \) is an allotetraploid species (Chalhoub et al., 2014). There are ten DELLA-encoding genes in the \( B. \) \( napus \) genome, including four homologs of \( B. \) \( tata \) DELLA \((\text{BnaRGA})\) (Gao et al., 2012). Mutants of these \( BnaRGAs \) were previously generated by CRISPR/Cas9-mediated gene editing (Yang et al., 2017). In the present study, we demonstrated that GA regulated FA biosynthesis through the \( BnaRGA-BnaLEC1 \) signalling cascade. \( BnaA6.RGA \) and \( BnaC7.RGA \) repressed FA biosynthesis by preventing \( BnaLEC1 \)s from activating FA-related gene expression. Our findings provide a novel mechanistic understanding of how phytohormones regulate FA production.

**Results**

**\( BnaRGAs \) are repressed during \( B. \) \( napus \) seed development**

FA content increases after exogenous GA treatment during the early stage of \( B. \) \( napus \) seed development (Huang et al., 2014). Thus, we hypothesized that the GA signalling repressors DELLA proteins play important roles in oil production in \( B. \) \( napus \). To investigate the potential roles of DELLA genes in \( B. \) \( napus \) oil biosynthesis, the expression patterns of these genes during seed development were examined by quantitative RT-PCR (qRT-PCR). The \( B. \) \( napus \) DELLA genes could be divided into three groups based on their expression patterns during seed development: genes whose expression decreased over time (the four homologous \( BnaRGA \) genes), genes whose expression increased over time \((BnaA5.RGL2 \) and \( BnaC5.RGL2)\), and genes whose expression decreased at the early stage and increased at the late stage of seed development \((BnaA10.RGL3 \) and \( BnaC9.RGL3)\) (Figure 1a). We then performed in silico analysis using available RNA-seq data to examine the expression patterns of DELLA genes in two inbred lines with an ~13% difference in SOC (Shahid et al., 2019). The expression patterns were consistent with the qRT-PCR results. The \( BnaRGAs \) were down-regulated during seed development from 11 to 44 DAP (days after pollination) (Figure 1b).

Interestingly, the four homologous \( BnaRGA \) genes were more strongly repressed in 1L99 (high-oil-content line, HOCCL) than in 1L363 (low-oil-content line, LOCL) (Figure 1b). These dynamic expression patterns of \( BnaRGA \) genes imply that \( BnaRGAs \) are likely to negatively regulate seed oil content.

**\( BnaRGAs \) negatively regulate seed oil content**

To further investigate the collective roles of the \( BnaRGA \) homologs in the regulation of SOC, two types of genome-editing mutants of \( BnaRGAs \) were generated by CRISPR/Cas9-mediated mutagenesis (Yang et al., 2017). The gain-of-function mutants of \( BnaA6.RGA \), encoding proteins containing a 2- or 4-amino acid deletion in the DELLA domain, were named \( bnaA6.rga-D \). The quadruple mutants, carrying loss-of-function mutations in all four \( BnaRGAs \), were named \( bnaRga \) (Yang et al., 2017). To determine whether \( BnaRGAs \) regulate SOC, firstly the seed-related agronomic traits were examined in the \( BnaRGA \) mutants and wild-type (WT) plants. The seed size and thousand-seed weight were significantly higher in \( bnaRga \) \((L27 \) and \( L46)\) and much lower in \( bnaa6.rga-D \) \((L4 \) and \( L6)\) compared to the WT (Figure 2a, b, Table 1). Then, the total seed oil and protein contents were measured by near-infrared reflectance spectroscopy. Compared to WT, \( bnaA6.rga-D \) showed a lower oil content and a higher protein content (Table 1). Although there was no difference in oil content between \( bnaRga \) and WT, the total FAs per seed of the \( bnaRga \) lines \( L27 \) and \( L46 \) \((1587.9 \) and \( 1644.3 \mu g)\) respectively were higher than that of the WT \((1459.0 \mu g)\) (Table 1). Then, the seed FA content was quantified by GC-MS. There was no significant difference in FA content among \( BnaRGA \) quadruple and single mutants in mature seeds (Figure S1), whereas the average SOC of \( bnaa6.rga-D \) \((36.95\%)\) was lower than that of the WT \((44.39\%)\) (Figure 2c). Subsequently, the SOC of \( bnaRga \) and \( bnaa6.rga-D \) was measured from 10 to 40 DAP, with 5-day intervals. Notably, the SOC of \( bnaRga \) was significantly higher than WT during the early stage \((10-25 \) DAP) (Figure 2c, Table 2). By contrast, \( bnaA6.rga-D \) had a significantly lower SOC than WT throughout seed development (Figure 2c, Table 2). These results indicate that \( BnaRGAs \) negatively regulate SOC.

A gain-of-function mutant of \( BnaC7.RGA \), \( ds-3 \), was previously obtained by screening a rapeseed EMS library in the \( HSS \) (huashuang5) background (Zhao et al., 2017). We therefore compared the SOC of \( ds-3 \) and \( HSS \). Similar to \( bnaa6.rga-D \), \( ds-3 \) had lower SOC (approximately 2.93% and 39.46% at 20 DAP and the mature stage, respectively) than \( HSS \) \((3.36\%\) and \(44.26\%)\) (Figure 2d). In general, the SOC of \( bnaRga \) and \( bnaA6.rga-D \) was measured from 10 to 40 DAP, with 5-day intervals. Notably, the SOC of \( bnaRga \) was significantly higher than WT during the early stage \((10-25 \) DAP) (Figure 2c, Table 2). By contrast, \( bnaA6.rga-D \) had a significantly lower SOC than WT throughout seed development (Figure 2c, Table 2). These results indicate that \( BnaRGAs \) negatively regulate SOC.

**Targeted mutations in \( BnaRGAs \) alter seed FA composition**

We then investigated whether \( BnaRGAs \) alter FA composition during seed development. Compared to WT, FA C18:1 level decreased by 3.6% and C18:2 level increased by 2.7% in mature \( bnaa6.rga-D \) seeds (Figure 3a). There was no significant difference in FA composition in mature \( bnaRga \) seeds vs. the WT (Figure 3a). The FA composition was also measured in seeds at 20 DAP. As shown in Figure 3b, gain-of-function mutation of \( BnaA6.RGA \) resulted in significantly higher C16:0, C18:2 and C18:3 contents and lower C18:1 content than the WT. Furthermore, C18:1 level increased by 7.6%, and C16:0, C18:2 and
C18:3 levels decreased by 2.0%, 4.4% and 0.79%, respectively, in bnarga compared to the WT (Figure 3b). Similar changes in FA composition were observed in ds-3, including a decreased proportion of C18:1 and increased proportions of C18:2 and C18:3 during different stages of seed development (Figure 3c,d).

Similar results were obtained for Arabidopsis mutants of RGA and its homolog GAI. Compared to the WT, total seed FA content decreased by 2.9% in gai (gain of GAI function in the Col-0 background), while rga24 (loss of RGA function in the Ler background) showed a slight increase in FA content (Figure S2a). The gai mutant also showed significantly reduced C18:0 and C18:1, and increased C18:2 compared to the WT (Figure S2b).

C18:0, C18:1 and C20:1 levels increased, while C16:0, C18:2, C18:3 and C20:2 levels dramatically decreased, in rga24 vs. the WT. The role of BnaRGAs in fat acid biosynthesis

Figure 1  Expression pattern of eight DELLA genes during different stages of seed development. (a) Relative gene expression of eight DELLA genes during the seed development was analysed by qRT-PCR; values were the means ± SE of three biological replicates. The gene expression levels of 10 D were set as 1, BnaActin acts an internal control. D, day after pollination. (b) The Log2FPKM values of eight DELLA genes during various stages of seed development of the HOCL (high-oil-content line) and LOCL (low-oil-content line).

Figure 2  Seed size, weight and oil content of bnarga, bnaa6.rga-D and WT. (a) The thousand-seed size of bnarga (L27 and L46), bnaa6.rga-D (L4 and L6) and WT. (b) Bar graph showed the 1000-seed weight of bnarga (L27 and L46), bnaa6.rga-D (L4 and L6) and WT. Data were means ± SD obtained from three biological experiments. (c) Bar graph showed the SOC of bnarga, bnaa6.rga-D and WT at 20 DAP and mature seeds. (d) Bar graph showed the SOC of ds-3, and HSS at 20 DAP and mature seeds. In (c) and (d), data were means ± SD obtained from three biological experiments detected by GC-MS. The data were analysed by Student’s t-test (*P < 0.05; **P < 0.01).
Table 1 Comparison for the quality and morphological of seed among the bnarga, bnaa6.rga-D and WT, respectively

| Traits                        | bnarga-L27 | bnarga-L46 | Westar     | bnaa6.rga-D-L4 | bnaa6.rga-D-L6 |
|------------------------------|------------|------------|------------|----------------|----------------|
| Oil content (%)              | 46.20 ± 0.57 | 45.97 ± 0.11 | 45.98 ± 0.44 | 40.94 ± 0.86** | 42.26 ± 0.96** |
| Protein (%)                  | 20.70 ± 1.13 | 20.84 ± 0.10* | 19.94 ± 0.36 | 23.88 ± 1.07*  | 23.36 ± 1.17*  |
| Seed size (mm²)              | 3.15 ± 0.14** | 3.24 ± 0.12** | 2.98 ± 0.13  | 2.61 ± 0.13**  | 2.47 ± 0.09**  |
| 1000-seed weight (g)         | 3.44 ± 0.02** | 3.58 ± 0.04** | 3.17 ± 0.03  | 2.75 ± 0.10**  | 2.77 ± 0.06**  |
| Total FAs (µg per mg)        | 462.0 ± 5.7  | 459.7 ± 1.1  | 459.8 ± 4.4  | 409.4 ± 8.7**  | 422.6 ± 9.7**  |
| Total FAs (µg per seed)      | 1587.9 ± 19.6** | 1644.3 ± 3.8** | 1459.0 ± 14.1 | 1124.4 ± 23.8** | 1169.3 ± 26.7** |

Data are means ± SD obtained from three biological experiments. Student’s t-test was used to calculate significance where * indicates P < 0.05 and ** indicates P < 0.01.

Table 2 Comparison for the seed oil content at different stages of seed development among the bnarga, bnaa6.rga-D and WT, respectively

| Seed    | bnarga-L27 | bnarga-L46 | Westar     | bnaa6.rga-D-L4 | bnaa6.rga-D-L6 |
|---------|------------|------------|------------|----------------|----------------|
| 10DAP   | 0.81 ± 0.08* | 0.79 ± 0.10* | 0.72 ± 0.08 | 0.62 ± 0.01*   | 0.63 ± 0.02*   |
| 15DAP   | 2.56 ± 0.17* | 2.72 ± 0.04* | 2.33 ± 0.06 | 1.85 ± 0.12*   | 1.87 ± 0.13*   |
| 20DAP   | 6.26 ± 0.38**| 6.50 ± 0.33**| 4.93 ± 0.17 | 2.66 ± 0.09**  | 2.5 ± 0.10**   |
| 25DAP   | 10.93 ± 0.59*| 10.41 ± 0.29*| 9.38 ± 0.56 | 7.56 ± 0.40**  | 8.65 ± 0.31**  |
| 30DAP   | 36.48 ± 1.14 | 36.33 ± 1.27 | 36.32 ± 0.59 | 34.03 ± 0.36*  | 30.67 ± 1.34** |
| 35DAP   | 41.62 ± 3.58 | 43.21 ± 0.73 | 43.58 ± 1.37 | 39.67 ± 0.97** | 39.49 ± 0.69** |
| 40DAP   | 45.47 ± 0.87 | 46.36 ± 1.14 | 44.55 ± 2.55 | 37.5 ± 1.13**  | 39.62 ± 1.16** |
| Mature  | 46.4 ± 0.54  | 45.37 ± 1.05 | 44.54 ± 3.65 | 36.83 ± 1.25** | 36.77 ± 2.51** |

Data are mean ± SE obtained from three biological experiments detected by GC-MS. Student’s t-test was used to calculate significance where * indicates P < 0.05 and ** indicates P < 0.01. DAP, day after pollination.

WT (Figure S2b). Overall, these results suggest that the roles of BnaRGA and its homologs in controlling the desaturation of oleic acid (C18:1) to linoleic acid (C18:2) are conserved.

BnARGAs alter the expression of FA-related genes during seed development

To determine the subcellular localization of BnaRGAs, we transiently expressed the BnaA6.RGA-GFP and BnaA9.RGA-GFP fusion genes in Arabidopsis mesophyll protoplasts. The fusion proteins were predominantly localized to the nuclei (Figure S3), which is consistent with their annotated functions as transcriptional regulators. We reasoned that transcriptomic analysis might provide additional insights into the role of BnaA6.RGA in controlling FA biosynthesis. Hence, RNA-seq analysis was performed to reveal the transcriptomic profiles of bnaa6.rga-D and WT seeds (10 and 20 DAP) (Figure 4a). We set FDR<0.05 and log₂FC (log₂) over >1 relative to WT as the cut-off threshold. In total, 1335 (1012 up-regulated and 323 down-regulated) genes were differentially expressed in bnaa6.rga-D at 10 DAP, and 3310 (2586 up-regulated and 724 down-regulated) genes were differentially expressed in this mutant at 20 DAP (Figure 2b, Table S1). 285 DEGs (differentially expressed genes) were identified in bnaa6.rga-D seeds at both stages (Figure 4c). GO (Gene Ontology) terms related to FA biosynthesis (GO: response to lipid, lipid biosynthesis process) and lipid metabolism (GO: lipid metabolic process, FA metabolic process) were enriched at both stages (Figure 4d, e). The expression of genes encoding glycolysis-related enzymes was suppressed in mutant seeds at 10 DAP (Figure 4f). Most genes encoding FA biosynthetic enzymes were also expressed at lower levels in bnaa6.rga-D than WT, including ACCase, MCMT, KASI/II/III, KAR, HAD, ENR, FATA, GPAT, PDAT and DGAT (Figure 4f, Table S2). qRT-PCR confirmed the transcript profiles of FA biosynthesis genes that were down-regulated in bnaa6.rga-D and up-regulated in bnarga (Figure S4).

The altered FA composition in the BnaRGA mutants prompted us to examine the expression of FA desaturase (FAD)-encoding genes in WT and BnaRGA mutants. Compared to WT, BnaFAD2 was significantly up-regulated in bnaa6.rga-D seeds at 20 DAP (Figure 4f). Consistently, during seed development, BnaFAD2 was up-regulated in bnaa6.rga-D and down-regulated in bnarga compared to WT (Figure S5).

GDSL-type lipases catalyse TAG degradation (Akoh et al., 2004); 222 genes with GDSL domains have been identified in B. napus (Table S3) (Karunarathna et al., 2020). Compared to WT, 13 and 24 GDSL genes were strongly up-regulated in bnaa6.rga-D at 10 and 20 DAP, respectively (Figure S6a). We selected several genes and verified their expression profiles by qRT-PCR; the results were consistent with the RNA-seq results (Figure S6b). The transcript levels of these genes were much lower in bnarga and dramatically higher in bnaa6.rga-D than in the WT, demonstrating that BnaRGAs positively regulate FA metabolism and reduce the SOC in B. napus.

BnaA6.RGA and BnaC7.RGA physically interact with BnaLEC1s

DELLA proteins usually function by interacting with other transcription factors (Davière and Achar, 2016). To further elucidate the roles of BnaRGAs in FA biosynthesis, we performed yeast two-hybrid assays to test protein interactions between
BnaA6.RGA and key Arabidopsis TAG biosynthesis-related transcription factors. Only AtLEC1 interacted with BnaA6.RGA (Figure S7). B. napus contains two LEC1 homologs (BnaA07g10770D and BnaC07g14150D) named BnaA7.LEC1 and BnaC7.LEC1, respectively (Figure S8a). We therefore examined the interactions between BnaRGA proteins and these two BnaLEC1 proteins via a yeast two-hybrid assay. Notably, among the four RGA proteins, only BnaA6.RGA and BnaC7.RGA interacted with the two BnaLEC1 proteins (Figure 5a). We then performed a bimolecular fluorescence complementation (BiFC) assay to examine the interaction between BnaLEC1s and the four BnaRGA proteins in vivo. YFP fluorescence was detected specifically in the nucleus only when BnaLEC1s-cYFP and BnaA6/C7.RGA-nYFP were expressed simultaneously in Nicotiana benthamiana leaves (Figure 5b). Pull-down assays further verified the interaction between BnaRGA proteins and BnaLEC1s, as GST-BnaLEC1s, but not GST alone, were precipitated with His-BnaA6.RGA in vitro (Figure 5c). Finally, the DELLA domain of BnaRGA was essential for the interaction between BnaRGAs and the BnaLEC1s (Figure 5d,e). However, there was a slight interaction between the N-terminal of the BnaRGAs and the C-terminal of the BnaLEC1s (Figure 5e). The results suggest that BnaRGAs might interact with BnaLEC1s and form protein complexes to regulate FA biosynthesis.

BnaRGAs regulate seed oil content via BnaLEC1s

LEC1 acts as a central regulator of FA biosynthesis in Arabidopsis (Mu et al., 2008). BnaLEC1s-GFP mainly localized to the nuclei, and some signal was also observed in the cytoplasm (Figure S8b). The expression patterns of the two BnaLEC1 homologs were similar, with expression increasing during seed development and reaching its highest level at 23 DAP (Figure S8c,d). Moreover, the BnaLEC1 genes were more highly expressed in the high-oil-content line 1L99 than in the low-oil-content line 1L363 (Figure S8c,d).

We then generated mutant lines of the BnaLEC1s by CRISPR/Cas9-mediated genome editing. Two single-guide RNAs (sgRNAs) were designed to target the two homologs of BnaLEC1 simultaneously (Figure 6a). Following genetic transformation into WT (Westar), eight mutants were isolated in the T0 generation. Substitutions, short deletions or short insertions at the sgRNA target sites were validated by sequencing (Figure 6b, Table 3). In addition, we separately transformed WT with the two homologs of BnaLEC1 driven by the CAMV 35S promoter (Figure 6c). The expression levels of BnaA7.LEC1 and BnaC7.LEC1 were ten-times higher in the transgenic lines than in WT (Figure 6c).

To characterize the roles of the BnaLEC1s in regulating seed oil content and FA composition, the T1 bnaa7.lec1 bnac7.lec1
double homozygous mutants (bnalec1, L7 and L9) and BnaLEC1-overexpression T1 lines (BnaA7.LEC1-ov, L16; and BnaC7.LEC1-ov, L24) were chosen for phenotypic characterization. The oil content of WT seeds was 45.97% of dry weight (Figure 6d). However, the seed oil contents of the bnalec1 mutants were approximately 34.02%, which were reduced by 11.95% relative to WT (Figure 6d). By contrast, the seed oil contents of the two overexpression lines were >50%, representing an increase of 4.65% relative to WT (Figure 6d). The overall changes in FA composition in the bnalec1 mutants showed a similar trend, with C18:1 level reduced by 5.68–6.73% and C18:2 level increased by 4.67%–5.62% relative to WT seeds (Figure 6e). By contrast, overexpressing the BnaLEC1s resulted in increased C18:1 level and decreased C18:2 level (Figure 6e). We then examined the expression patterns of FA biosynthesis-related genes in bnalec1, BnaLEC1-ov and WT seeds at 20 DAP. Consistently, BnaABI3 and FA biosynthesis enzyme-encoding genes (e.g. ACCase, MCMT and KASI) were significantly down-regulated in bnalec1 and up-regulated in BnaLEC1-ov at 20 DAP (Figure S9). The relative expression levels of BnaFAD2 were generally higher in bnalec1 and lower in BnaLEC1-ov than in the WT (Figure S9).
To further investigate the genetic relationship between BnaRGAs and BnaLEC1s, the "bnarga bnalec1" sextuple mutants were brought out for genetic analysis (Figure 7a). The oil content of F2 sextuple mutant ("bnarga bnalec1", L8 and L16) seeds was approximately 39.61%, representing an increase of 3.46–5.59% relative to "bnalec1" seeds (Figure 7b). However, there was no obvious difference in FA composition between "bnalec1" and "bnarga bnalec1" seeds (Figure 7c). In addition, the expression patterns of FA biosynthesis genes were similar between "bnalec1" and "bnarga bnalec1" sextuple mutants (Figure 7d). These results demonstrate that BnaLEC1s are epistatic to BnaRGAs in regulating FA biosynthesis.

BnaRGAs repress BnaLEC1-mediated BnaA3.ABI3 expression

LEC1 regulates SOC by mediating the expression of genes involving in FA biosynthesis (Mu et al., 2008). The relative expression levels of BnaABI3 were generally higher in bnarga and lower in bna6.rga-d than in the WT (Figure 8a,b), suggesting that BnaRGAs repress BnaLEC1-mediated BnaABI3 expression. To investigate this hypothesis, the dual-luciferase reporter assays were performed to examine the transcriptional regulation of BnaABI3 by BnaLEC1s-BnaRGAs. The reporter vector contained a firefly luciferase gene driven by the BnaA3.ABI3 promoter and a renilla luciferase (REN) gene driven by the CaMV 35S promoter. BnaA6.RGA, BnaA9.RGA, BnaA7LEC1 and BnaC7.LEC1 were each driven by the CaMV 35S promoter in independent effector vectors (Figure 8c). The LUC activities were measured using different combinations of reporter and effector vectors. Compared with the expression of pBnaA3.ABI3-LUC only, LUC activity was approximately twice greater when this vector was co-expressed with BnaA7.LEC1 or BnaC7.LEC1 (Figure 8d). Furthermore, when BnaA6.RGA was expressed together with BnaA7.-LEC1 or BnaC7.LEC1, pBnaA3.ABI3-LUC activity decreased by 1.7- or 1.8-fold, respectively (Figure 8d). There was no significant decrease in LUC activity when BnaA9.RGA was co-expressed with any of the BnaLEC1s (Figure 8d). Collectively, these results suggest that BnaA6.RGA forms complexes with BnaLEC1s to suppress the expression of BnaA3.ABI3.

Association analysis of BnaRGAs and BnaLEC1s with SOC

To further validate the roles of BnaRGAs and BnaLEC1s in regulating SOC, we performed an association analysis using a set of 505 inbred lines collected from different geographical regions (Tang et al., 2020b). The SOC varied from 34.88% to 55.25%, and 36 and 4 single nucleotide polymorphisms (SNPs) were detected in BnaA6.RGA and BnaC7.RGA, respectively, by resequencing. However, there were no significantly associated SNPs in BnaA6.RGA or BnaC7.RGA (Figure S10a,b), suggesting that BnaRGAs were not selected during B. napus domestication. Notably, we identified a significantly associated SNP in the promoter region of BnaA7.LEC1, named BnvaA0710128806.

Figure 5 BnaRGAs physically interacted with BnaLEC1. (a) BnaA6.RGA and BnaC7.RGA interacted with BnaA7.LEC1 and BnaC7.LEC1 in the Y2H assay. (b) BnaA6.RGA and BnaC7.RGA interacted with BnaA7.LEC1 and BnaC7.LEC1 in the BiFC assay performed in the N. benthamiana leaves. Scale bars: 50 μm. Images were acquired by confocal microscope using the identical settings. YFP, yellow fluorescent protein; auto, chloroplast auto fluorescence; bright, bright field; merge, the figure merged by YFP, and auto. (c) BnaA6.RGA interacted with BnaLEC1s in the pull-down assay. Glutathione beads loaded with recombinant GST or GST-BnaLEC1s were incubated with 6×His-BnaA6.RGA. After incubation, the input fractions and GST pull-down fractions were detected by Western blotting with antibodies against GST and 6×His. The specific bands of His-BnaA6.RGA, GST-BnaLEC1s and GST were labelled by asterisk, triangle and arrow, respectively. (d) Schematic diagram showed the domain structures of BnaRGAs, BnaLEC1s and their various truncated versions. (e) Yeast two-hybrid assays showed the interactions between different truncation proteins of BnaLEC1s and BnaRGAs. Transformed yeast cells were grown on SD-Trp-Leu-His-Ade and SD-Trp-Leu media.
which explained 2.1% of SOC variation in this population (Figure S10c). The population was classified into two groups based on the genotype of SNP BnvaA0710128806 (GG/AA). Lines with the genotype GG showed higher oil content than lines with the genotype AA \((P = 9.5 \times 10^{-4})\) (Figure S10d), suggesting that BnaA7.LEC1 is associated with SOC in the natural population. Furthermore, a specific PCR marker was developed based on this SNP to distinguish between lines with these two genotypes (GG/AA) (Figure S10e). This marker could be used to select materials with high-oil content for breeding.

**GA biosynthesis genes are up-regulated during seed development**

In plants, DELLA proteins are degraded when GA increase (Ito et al., 2018). Therefore, we examined the expression levels of GA biosynthesis genes during seed development in the B. napus Transcriptome Information Resource (BnTIR; http://yanglab.hzau.edu.cn). The expression of genes involved in bioactive gibberellin biosynthesis, such as GA20ox4, KAO2 and KS, gradually increased during seed development (14-44 DAP) (Figure S11), which was correlated with FA accumulation (Tang et al., 2020b). This consistency between the expression patterns of GA biosynthesis genes and FA accumulation suggests that GA positively regulates SOC, and it further supports the negative role of BnaRGA in FA biosynthesis.

**Expression of FA biosynthesis and GDSL genes in response to gibberellin treatment**

Finally, to examine the role of GA in FA biosynthesis in B. napus, we treated B. napus ‘Westar’ siliques with GA3 after self-pollination. At 20 DAP, the siliques were longer and produced larger seeds after GA treatment (Figure S12a, b). After GA treatment, the transcript levels of BnaABI3 and genes encoding FA biosynthesis enzymes (ACCase, ENR, DGAT1 and PDAT) were increased, and BnaFAD2 transcript levels were generally declined (Figure S12c). In addition, GDSL genes were repressed by GA treatment (Figure S12d). These results suggest that GA positively regulates SOC during early seed development.

**Discussion**

*Brassica napus*, the second largest source of edible oils worldwide, has great potential for further increases in SOC (Chew, 2020). The FA biosynthetic pathway in *B. napus* is known, and several materials with high-oil content have been generated via breeding (Hua et al., 2016). However, how phytohormones regulate FA production in *B. napus* is still unknown. In this study, we demonstrated that BnRGAs, which function as conserved repressors of GA signalling, negatively regulate FA biosynthesis by interacting with BnaLEC1s in
Table 3 Genotypic analysis of BnaLEC1 mutants in T0 and T1 generations

| Plant ID       | Generation | Genotype at targets of BnaA7.LEC1 | Genotype at targets of BnaC7.LEC1 |
|----------------|------------|-----------------------------------|-----------------------------------|
|                |            | sgRNA1   | sgRNA2   | sgRNA1   | sgRNA2   |
| bnalec1-L3     | T0         | Hetero   | wt       | wt       | wt       |
| bnalec1-L3-2   | T1         | Homo(1s) | wt       | wt       | wt       |
| bnalec1-L3-3   | T1         | Homo(1)  | wt       | wt       | wt       |
| bnalec1-L4     | T0         | wt       | wt       | Hetero   | Hetero   |
| bnalec1-L4-2   | T1         | wt       | wt       | Homo(1)  | Homo(1)  |
| bnalec1-L6     | T0         | wt       | Homo(1s)| wt       | wt       |
| bnalec1-L6-2   | T1         | wt       | Homo(1s)| wt       | wt       |
| bnalec1-L7     | T0         | Hetero   | wt       | Hetero   | wt       |
| bnalec1-L7-2   | T1         | Homo(1d) | wt       | Homo(1)  | wt       |
| bnalec1-L9     | T0         | Hetero   | Hetero   | wt       | wt       |
| bnalec1-L9-2   | T1         | Homo(1d) | wt       | Homo(1)  | wt       |
| bnalec1-L9-3   | T1         | Homo(1d) | wt       | Homo(1)  | wt       |
| bnalec1-L11    | T0         | wt       | Homo(1)  | wt       | Homo(1)  |
| bnalec1-L11-2  | T1         | wt       | Homo(1)  | wt       | Homo(1)  |
| bnalec1-L12    | T0         | Hetero   | Hetero   | wt       | wt       |
| bnalec1-L12-2  | T1         | wt       | Homo(1)  | wt       | wt       |
| bnalec1-L12-3  | T1         | Homo(1)  | Homo(1s)| wt       | wt       |
| bnalec1-L13    | T0         | wt       | Homo     | Hetero   | Hetero   |
| bnalec1-L13-2  | T1         | wt       | Homo     | wt       | Homo(1s)|
| bnalec1-L13-3  | T1         | wt       | Homo     | Homo(1)  | Homo(2) |

Hetero, heterozygous; Homo, homozygous; wt, no mutations were identified; #d, # of bp deleted from a target site; #i, # of bp inserted at target site; #s, # of bp substitute at target site.

B. napus, providing novel insights for the genetic improvement of SOC in B. napus.

BnaRGAs suppress FA biosynthesis in B. napus

The de novo FA biosynthetic pathway in Arabidopsis has been described in detail (Baud and Lepiniec, 2009). Three major steps in this pathway, condensation, elongation and desaturation, coordinately determine SOC and FA composition in Arabidopsis seeds (Bates et al., 2013; Slabas and Fawcett, 1992; Troncoso-Ponce et al., 2016). Several transcription factors, such as LEC1, AB13, FUS3, LEC2 and WR11, also play important roles in regulating seed oil accumulation by regulating FA biosynthesis genes (Santos-Mendoza et al., 2008; To et al., 2006). Disrupting these genes usually results in changes in oil content and FA composition (Tan et al., 2011; Tang et al., 2018). Analysis of transcriptomes generated from B. napus seed tissues suggests that homologs of these FA biosynthesis and transcription factor genes play conserved roles in oil production and determining FA composition in B. napus (Tan et al., 2011). Many SOC- and FA-related QTLs (quantitative trait loci) have been identified by GWAS (genome-wide association studies) (Wang et al., 2018). However, few genes involved in regulating FA biosynthesis in B. napus have been functionally studied, except for BnaLEC1 and BnaWRI1 (Elahi et al., 2016; Wu et al., 2014). The gain-of-function mutants bnaa6.rga-D and ds-3 showed significantly reduced SOC. By contrast, SOC was increased in the loss-of-function quadruple mutant bnaraga only during earlier seed development. These results indicate that the BnaRGAs act as repressors to regulate SOC. Interestingly, the FA composition was altered in the seeds of these mutants, with increased linoleic acid and decreased oleic acid contents in bnaa6.rga-D and ds-3, and decreased linoleic acid and increased oleic acid contents in bnaraga at 20 DAP (Figure 3), suggesting that BnaRGAs are also involved in regulating FA composition.

Several RGA homologs have been functionally characterized in various plant species (Oh et al., 2014; Xu et al., 2016), and a few have been shown to regulate SOC and FA composition. In Arabidopsis, the total FAs per milligram of seeds is lower in DELLA quadruple mutants (gai-t6 rga-t2 rgl1-1 rgl2-1) than in WT (Chen et al., 2012). During B. napus seed development, the expression of the RGA homologs (BnaRGAs) was suppressed, while the expression of the other DELLA protein-encoding genes was enhanced (Figure 1a), indicating that BnaRGA proteins act differently from other DELLA proteins in regulating SOC in B. napus SOC. Interestingly, BnaRGA genes were more strongly repressed in a line with high-oil content and less strongly repressed in a line with low-oil content (Figure 1b). These expression patterns imply that BnaRGAs are negatively associated with SOC.

In Arabidopsis, DELLAs suppress the expression of GDSL family genes in imbibed seeds to increase SOC (Chen et al., 2012). Overexpression of SFAR4 (SEED FATTY ACID REDUCER4) in Arabidopsis resulted in reduced total seed FA content (Huang et al., 2015a), while knocking out the GDSL family genes SFAR1, SFAR4 and SFAR5 in B. napus significantly increased the SOC (Kuraranatha et al., 2020), suggesting that the role of SFARs in FA catabolism is likely conserved in B. napus. Compared to WT, the expression of the GDSL family genes BnaAnng05710D, BnaA09g15250D and BnaA06g01430D was greatly enhanced in bnaa6.rga-D and suppressed in bnaraga (Figure S6), demonstrating that BnaRGAs reduce SOC by positively regulating the expression of GDSL family genes in B. napus. However, other DELLA protein-encoding genes, that is BnaRGL2s and BnaRGL3s, were induced during the seed development, pointing to the diverse roles of DELLA proteins in controlling GDSL family gene expression during different seed development stages. Unlike the GDSL family genes, other FA biosynthesis genes were suppressed in bnaa6.rga-D and up-regulated in bnaraga (Figure 4f, Figure S4). The specific expression patterns of these FA biosynthesis genes might also contribute to their distinct roles in regulating SOC.

FAD2 is an endoplasmic reticulum desaturase that is essential for polyunsaturated lipid biosynthesis (Dar et al., 2017). Mutants of BnaFAD2 show reduced FAD2 activity, resulting in decreased linoleic acid and increased oleic acid content in B. napus (Huang et al., 2020). Here, we revealed that the expression of BnaFAD2 was highly induced in bnaa6.rga-D and repressed in bnaraga (Figure S5), suggesting that the changes in C18:1 and C18:2 contents in BnaRGAs mutants are due to the altered regulation of BnaFAD2 expression.

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BnaRGAs directly interact with BnaLEC1s to regulate SOC and FA composition in *B. napus*

DELLA proteins lack a DNA-binding domain, requiring them to form complexes with DELLA-interacting proteins (DIPs) to regulate plant development, stress responses and other biological processes (Van De Velde et al., 2017). In Arabidopsis, the DELLA-LEC1 complex regulates late embryogenesis by mediating auxin biosynthesis (Hu et al., 2018), but this interaction mechanism has not yet been clearly defined in plants harbouring duplicated genes. *B. napus* has four BnaRGA proteins with high sequence similarity (Wu et al., 2020). We previously revealed that the four BnaRGA proteins interact with BnaA10.ABF2 to enhance drought tolerance, pointing to a conserved mechanism among BnaRGAs that functions in the drought response (Wu et al., 2020).

Although the expression of all four *BnaRGA* genes was suppressed during seed development, only two of their encoded proteins, BnaA6.RGA and BnaC7.RGA, physically interacted with the two BnaLEC1s (Figure 5). Furthermore, BnaA6.RGA, but not BnaA9.RGA, significantly inhibited the expression of downstream genes of BnaLEC1 (Figure 8d). We propose that BnaA9.RGA and BnaC9.RGA interact with other proteins to mediate FA biosynthesis. These findings reveal that gene duplication of *BnaRGAs* plays an essential role in FA biosynthesis in *B. napus* via divergent mechanisms. The detailed functions of the different BnaRGA genes in FA biosynthesis remain to be explored.

**Figure 7** BnaLEC1s was epistatic to BnaRGA in FA biosynthesis. (a) The genotype of the *bnarga bnalec1* sextuple mutants in F2 generation. The protospacer adjacent motif (PAM) region was marked in green. The sgRNA was denoted in red. The mutation sites were indicated in blue. (b) Bar graph showed the oil content of *bnalec1*, *bnarga* and WT. (c) Bar graph showed the FA composition of *bnalec1*, *bnarga* and WT. (d) Bar graph showed the expression levels of FA biosynthesis genes in *bnalec1* (L7), *bnarga* (L8) and WT seeds at 20 DAP. The gene expression level of WT was set as 1. *BnaActin* acted an internal control. In (b) and (c), data were means ± SD obtained from three biological experiments detected by GC-MS. In (b), (c) and (d), Student’s *t*-test was used to calculate significance, and letters indicate statistically differences between b: *bnalec1* vs WT (Westar); c: *bnarga* vs WT (Westar); and d: *bnarga* vs *bnalec1* at *P* < 0.01.

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acquired different roles in regulating SOC in different species over the course of evolution.

In Arabidopsis, more than 50% of genes involved in plastid FA biosynthesis are regulated by LEC1 (Mu et al., 2008), indicating that LEC1 is a key transcriptional activator that regulates SOC. Knocking out and overexpressing BnaLEC1 resulted in decreased and increased SOC, respectively (Figure 6), indicating that LEC1 homologs play conserved roles in positively regulating SOC. Notably, FA composition is also altered in bnalec1 and bnarga seeds (Figure 3 and 6). In addition, BnaLEC1 negatively regulated the mRNA accumulation of BnaFAD2 (Figure S9), while BnaFAD2 was up-regulated in bnaa6.rga-D and down-regulated in bnarga (Figure S5). These results indicate that BnaLEC1 repress the expression of BnaFAD2 and that BnaRGA might reduce this suppressive effect. Since LEC1 also forms protein complexes with other transcription factors (Jo et al., 2019), one possibility is that other transcriptional regulators might interact with the RGA-LEC1 complex to further control FA composition. However, no obvious difference in FA composition was detected between bnalec1 and bnarga bnalec1 sextuple mutants (Figure 7c), demonstrating that BnaLEC1s are epistatic to BnaRGAs in regulating FA composition.

The developmental stage-dependent function of BnaRGAs in regulating SOC and FA composition

Seed formation comprises two major phases: morphogenesis and maturation (Baud and Lepiniec, 2009). Aborted embryo development is accompanied by the failed accumulation of seed storage compounds (Chaudhury et al., 2001), which could be attributed to the partially overlapping and synergistic functions of LAFL proteins (LEC2, ABI3, FUS3 and LEC1) (Fathi et al., 2016). BnaRGAs repressed SOC accumulation by repressing BnaLEC1 activity. DELLA-LEC1 complexes are also essential for embryogenesis in Arabidopsis (Hu et al., 2018). These findings indicate that DELLA-LEC1 mediate both seed morphogenesis and maturation.

Figure 8 Transcription regulation of BnaA3.ABI3 by BnaLEC1s was repressed by BnaA6.RGA. (a) Bar graph showed the TPM value of two homologues of BnaABI3 from transcriptome of WT and bnaa6.rga-D at 10 and 20 DAP seeds. (b) The expression of BnaABI3 in bnarga, bnaa6.rga-D and WT from 10 to 40 DAP (with 5-day interval) examined by qRT-PCR. The gene expression levels of WT were set as 1. BnaActin acted an internal control. (c) Schematic representation of the constructs used for the dual-luciferase assay. The reporter construct contained the firefly luciferase driven by BnaA3.ABI3 promoter, and the Renilla luciferase (REN) was driven by the CaMV 35S promotor. The effector constructs contained BnaA6.RGA, BnaA9.RGA and BnaLEC1s driven by the CaMV 35S promoter, respectively. (d) Bar graph showed the LUC/REN ratios in the dual-luciferase assay. In (d), letters indicate statistically differences between b: co-transformed effectors (BnaLEC1s or BnaRGAs) with reporter (pBnaA3.ABI3:LUC) vs control (pBnaA3.ABI3:LUC); c: co-infiltrated effectors (BnaRGAs + BnaLEC1s) with reporters vs co-infiltrated effectors (BnaLEC1s) with reporters; and d: co-infiltrated effectors (BnaRGAs + BnaLEC1s) with reporters vs co-infiltrated effectors (BnaRGAs) with reporters at P < 0.05 (Student’s t-test). In (a) and (b), data were means ± SD obtained from three biological experiments. Student’s t-test was used to calculate significance where * indicated P < 0.05 and ** indicated P < 0.01.

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providing clues about the intimate link between these two phases of seed development.

On the other hand, seed development is an intricate process that is precisely controlled via a complex regulatory network during different stages (Gupta et al., 2017). Following embryo morphogenesis in B. napus, total FAs start to accumulate around 20 DAP, followed by a period of rapid accumulation after 30 DAP (Shahid et al., 2019). During seed development, GA biosynthesis gene expression gradually increased (Figure S11) and was negatively associated with the expression of BnaRGAs. Furthermore, the peak expression of BnaLEC1s occurred at 20-25 DAP (Figure S8c and S8d). Consistent with these expression patterns, the SOC and expression levels of FA-related genes were significantly reduced in BnaRGA gain-of-function mutants and increased in bnarga mutants during the earlier seed development stage. These results suggest that the RGA-LEC1 complex also functions in FA biosynthesis after embryogenesis. In mature Arabidopsis seeds, the expression of GDSL-type SFAR genes is suppressed by DELLA proteins (Chen et al., 2012), implying that the roles of BnaRGAs in regulating SOC and FA composition are stage dependent.

The use of BnaRGAs and BnaLEC1s for B. napus improvement

As SOC is the most important agronomic trait of B. napus, various SOC-related QTLs have been identified by traditional QTL mapping and GWAS (Jiang et al., 2014). However, given the complexity of seed oil biosynthesis, additional loci are likely to be identified and could be utilized for breeding high SOC B. napus. BnaRGAs and BnaLEC1s made only small contributions to SOC in the natural population analysed in this study. However, the phenotypic variation of this population may not have been wide enough to uncover the full potential of these genes, suggesting that BnaRGAs and BnaLEC1s are potentially useful loci for breeding high-oil B. napus.

Taken together, our results reveal that BnaRGA proteins play essential roles in FA biosynthesis by interacting with BnaLEC1s. We propose a working model for the roles of BnaRGAs in mediating time-dependent FA synthesis in B. napus (Figure 9). According to this model, during the early stage of seed development, when GA levels are low, BnaRGAs form a complex with BnaLEC1s and block their DNA-binding capacity, leading to repressed fatty acid synthesis. In addition, BnaRGAs activate the expression of GDSL lipases, which further represses SOC accumulation. During seed development, GA levels gradually increase, which triggers the degradation of BnaRGAs to relieve their repression of BnaLEC1s. Simultaneously, BnaLEC1s activate the expression of FA-related genes to promote FA biosynthesis. By contrast, the expression of BnaFAD2 is repressed by BnaLEC1s. The resulting reduction in FAD2 activity leads to reduced oleic desaturation, thereby facilitating the accumulation of oleic acid and reducing the level of linoleic acid. Our findings shed light on the essential roles of BnaRGA-BnaLEC1 in seed oil accumulation and FA composition, and they provide valuable information for increasing oil content and manipulating FA composition in B. napus.

Material and methods

Plant materials and growth condition

The B. napus recessive single mutant, bnaa6.rga, bnaa7.rga, bnaa9.rga, bnaac9.rga, quadruple mutant bnarga, double mutant bnalec1 and gain-of-function mutant bnaa6.rga-D were generated by CRISPR/Cas9 technology in B. napus (Westar) (Yang et al., 2017). ds-3 was obtained by previous study (Zhao et al., 2017). The bnarga bnalec1 sextuple mutants were obtained by crossing between quadruple mutants bnarga and double mutants bnalec1. All mutants and wild-type (WT) seeds were soaked in water for 7 days and then placed in soil and grown in the transgenic plant field at Huazhong Agricultural University.

Arabidopsis thaliana gai, rga24 and their relative VTE were obtained from previous study (Wang et al., 2020). All mutants and WT were grown in greenhouse under a light intensity of 100 μmol/m2/s–1 with a 16/8 h light/dark photoperiod at 22 °C.

Plasmid construction

The genes were amplified from Arabidopsis and B. napus cDNA library by PCR using gene-specific primers. For the yeast two-hybrid assays, the CDS sequences of ATL1C1, ATWRI, ATFUS3, BnaLEC1s and BnaRGAs or the truncated versions of BnaLEC1s and BnaRGAs were amplified by PCR and cloned into the vector pGADT7 or pGBK7 (Clontech, USA), respectively. For the BiFC assay, the CDS of BnaRGAs were inserted into the vector pFGC-YN173 fused with the N terminus of YFP, and the CDS of BnaLEC1s was inserted into the vector pFGC-YC155 fused with the C terminus of YFP. For the pull-down assay, the CDS of BnaLEC1s and BnaRGAs were inserted into pGEX-4T-3 (NOVA-GEN, Darmstadt, Germany) or pFastBac-His (Invitrogen, CA, USA) vector for the expression of GST or His fusion proteins, respectively. For the transient expression assay, ~1.5 kb Bnaa3.AB3 promoter was inserted into the vector pGreenII 0800-LUC as the reporter, while the CDS of BnaRGAs and BnaLEC1s were inserted into the pGreenII 62-SK vector as effectors. To generate BnaLEC1s double mutants, the GENE-sgRNA plant expression vectors were constructed as previously described with minor modifications (Xing et al., 2014). Briefly, two sgRNAs were designed to target the homologues of BnaLEC1s second exon by CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) (Lei et al., 2014). The sgRNAs cassettes were amplified by PCR using pcBC-DT13 as template, and the PCR fragments were then inserted into pKSE401G by Golden Gate Assembly (Gao et al., 2013). To generate pri101-BnaLEC1s-GFP vectors, the CDS of two BnaLEC1s homologues were amplified by PCR and then cloned into pri101-GFP vector (An et al., 2018). All primers were listed in Table S4.

Plant transformation and genotyping

The procedure of Agrobacterium-mediated B. napus transformation was carried out as previously described (Dai et al., 2020). To genotype the CRISPR/Cas9-mediated mutation, CTAB method was used to extract the genomic DNA (Molecular Cloning 4th edition). The target-specific were amplified by PCR, and amplimers were sub-cloned into the pMD18-T vector (6011, Takara, Tokyo, Japan), at least six clones were individually sequenced using Sanger sequencing for every accession. The primers were listed in Table S4.

NIRS analysis and GC-MS of FA accumulation in seed

NIRS analysis was performed by a Foss NIRSystems 5000 near-infrared reflectance spectrocope to detect SOC, which was followed as previous report (Tang et al., 2020a). FA and oil contents were analysed by GC-MS as previous describe (Lu et al., 2016). Oil content was expressed as percentage of the dry weight, and FA composition was calculated as the percentage of...
the total FA. At least five replications of each accession were used for analysis.

**RNA isolation and transcriptome sequencing**

The seeds at 10 and 20 DAP were collected and frozen immediately into liquid nitrogen. Total RNA was extracted using an RNeasy Prep pure plant Kit (TIANGEN Cat. DP441) following the manufacturer’s instructions. Construction of Illumina libraries and sequencing were completed by GENOSEQ. Totally, 319,050,468 high-quality raw reads from 12 samples were generated by Illumina sequencing platform. Before mapping to the reference genome, all raw reads were filtered using trim_galore (version 0.6.1). All reads which include adapters, low-quality sequences (below 20) were filtered. Subsequently, all filtered clean reads were mapped to the B. napus reference genome (Brassica_napus_v4.1.chromosomes; https://www.genoscope.cns.fr/brassica_napus/data/) by hisat2 (version 2.1.0) with default parameters. Alignment sam files were then transformed to bam files by samtools (version 1.9) (Li et al., 2009). Reads count numbers were calculated by featureCounts (version 1.6.4) (Liao et al., 2014). Different expression genes were analysed by DESeq2 packages using the former results of featureCounts. Then, transcript abundance was measured as a TPM value using TBtools (version 1.076) (Chen et al., 2020). Expression levels (Log2TPM value) of genes were visualized using a heatmap (TBtools version 1.076). For the FA biosynthesis genes, the final TPM values were integrated all paralogues of FAs biosynthesis enzyme-encoding genes followed with previous describe (Lu et al., 2018). All the TPM values of FAs biosynthesis enzyme-encoding genes were listed in Table S2. Three valid biological replicates were carried out for the transcriptomic analysis.

**Quantitative reverse-transcription PCR**

The seeds from different material were collected from 10 to 40 DAP (with 5-day intervals) after self-pollination. The cDNA was synthesis using a Transcript RT kit (TaKaRa, Cat. #RR047A). For qRT-PCR, a total volume of 10 μL reaction mixtures was used containing 5 μL of 2× SYBR Green Master Mix (963400, TOYOBO, Tokyo, JAPAN), 4.6 μL of 100× diluted cDNA and 0.2 μL of each primer. Amplification was performed using a CFX384 Real-Time System (786BR04835, BioRad, CA, USA). The amplification program was performed as previous describe (Wu et al., 2020). The expression level of each gene was calculated using the 2^(-ΔΔCT) method (Livak and Schmittgen, 2001). The primers were designed to target the conserved region of paralogues of FA biosynthesis relative genes. All analyses were repeated at least three times. The primers used for qRT-PCR were listed in Table S5.

For GA treatment, the wild-type (Westar) siliques were sprayed with 10 mM GA3 or 10% ethanol (control) for 15 days (with 1-day intervals) after self-pollination. At 20 DAP, the seeds were collected and stored at −80 °C for RNA extraction. Three valid biological replicates were carried out for the analysis.

**Yeast two hybrid**

The yeast Gal4 system was employed for two-hybrid analysis following yeast transformation handbook (Yeast Transformation System 2, Clontech, CA, USA). The protein interaction assay was performed as previous described (Wu et al., 2020).

**Bimolecular fluorescence complementation and subcellular localization assay**

The Bimolecular fluorescence complementation (BiFC) assay and protein subcellular localization analysis in N. benthamiana or Arabidopsis mesophyll protoplasts were performed as previously described (Miller et al., 2015; Zheng et al., 2016). In brief, in N. benthamiana, Agrobacterium tumefaciens (strain GV3101) cells containing the desired constructs were co-infiltrated into 4- to 5-week-old N. benthamiana leaves with infiltration buffer (50 mM MES, pH = 5.6, 2 mM Na3PO4, and 1 mM acetosyringone). The final concentrations of the bacteria were adjusted to an OD600 = 0.6 for each construct. Infected leaves were analysed at 48h after infiltration. In Arabidopsis mesophyll protoplasts, BnaRGA-GFP or Ghd7-RFP fusion genes were first transformed...
into *Arabidopsis* mesophyll protoplasts as previously described (Huang et al., 2015b), infected cells were analysed at 16h after transfection. YFP fluorescence (Excitation /Emission wavelength: 514 nm/527 nm), GFP fluorescence (Excitation /Emission wavelength: 488 nm/509 nm) and RFP fluorescence (Excitation / Emission wavelength: 559 nm/583 nm) were observed under a fluorescence microscope (SPX8, Leica, Wetzlar, Germany).

**Protein expression and purification**

GST-LEC1s plasmids were transformed into Escherichia coli BL21 cells and incubated overnight to induce expression of recombinant proteins in the presence of 0.1mM IPTG at 16 °C. The purification of GST fusion proteins was performed by Mag-Beads GST Fusion Protein Purification (BBI Life Science NO. C650031) and stored in aliquots at −80 °C. Recombinant His-BnaA6.RGA protein was expression in insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the described previously (Park and Shin, 2015). The His fusion proteins were further purified using the Mag-Beads His-Tag protein purification (BBI Life Science NO. C650033) and stored in aliquots at −80 °C.

**Pull-down assay**

For pull-down assay, the purified equal volumes of GST or GST-BnaLEC1s were incubated with Glutathione Magarose beads (BBI Life Science NO. C650031) at 4°C for 3 h. After incubation, His-BnaA6.RGA fusion protein was added to the target protein magnetic bead complex, and it was incubated for another 2 h at 4 °C. The bead-bound proteins were washed with PBSF five times. After washing, the bound proteins were eluted and separated using SDS-PAGE, and then detected with anti-GST (D190101-0100, BBI) or anti-His (D191001-0100, BBI) antibodies.

**Dual-luciferase assay**

For dual-luciferase assay, the effector and reporter constructs were first transformed into *Arabidopsis* mesophyll protoplasts as previously described (Huang et al., 2015b). Then, dual-luciferase assay was followed the instruction of Dual-Luciferase® Reporter Assay System (E1910, Promega, WI, USA). The data were represented as the ratio of firefly to Renilla luciferase activity (Fluc/Rluc). Each data point consisted of at least three replicates, and three independent experiments were performed for each assay.

**Association analysis of BnaRGA and BnaLEC1**

A total of 505 *B. napus* accessions collected from different research institutions were grown in Wuhan for two growth seasons (2012–2013 and 2013–2014). Mature open-pollinated seeds of the natural population were harvested and desiccated for the analysis of SOC by a Foss NIRSystems 5000 near-infrared reflectance spectroscope, and the polymorphic SNPs of BnaRGA and BnaLEC1s were genotyped by genomic DNA re-sequencing (Tang et al., 2020a). The efficient Mixed-Model Association eXpedited (EMMAX) analyses were used for genome-wide association analysis.

**Molecular marker analysis**

A dominant molecular marker was developed to distinguish two different genotypes of SNP BnaA0710128806. For genotyping, genomic DNA was extracted, and the target sequences were amplified by PCR using the following cycling conditions: 95 °C for 5 min; 32cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 20 s; and 72 °C for 5 min. Approximately 5 μL of the amplified products was separated by electrophoresis in 2% (w/v) agarose gels (TsingKE NO. TSJ001). The primers were listed in Table S4.

**Statistical analyses**

Statistical analysis was performed to identify significant between genotypes, using Student’s t-test, at *P* values < 0.05 or < 0.1.

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**Author contributions**

C.D. C.M., and G.Y. designed the research and wrote the manuscript. G.Y., P.Y. and X.T. performed the experiments and analyzed the data. L.G. provided GC-MS support and *B. napus* resequencing data. K.L. provided ds-3 mutant. J.T., J.S., B.Y., J.W., and T.F. provided lab support. All authors read and approved the manuscript.

**Conflict of interest**

The authors have no conflicts of interest to declare.

**Data Availability Statement**

Transcriptome data were deposited in NCBI under BioProject ID: PRJNA699093.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Seed oil content and 1000-seed weight of BnaRGA recessive mutants and WT mature seeds.  
Figure S2 Seed oil content and FAs composition in gai, rga24 and WT (Col-0 and Ler) mature seeds.  
Figure S3 Subcellular localization of BnaA6.RGA and BnaA9.RGA in Arabidopsis thaliana protoplasts.  
Figure S4 Expression of FA biosynthesis genes in bnarga, bnaa6.rga-D and WT.  
Figure S5 Expression of BnaFAD2 in bnarga, bnaa6.rga-D and WT.  
Figure S6 Expression of GDSL family genes in bnarga, bnaa6.rga-D and WT.  
Figure S7 BnaA6.RGA interacted with AtLEC1 in the Y2H assay.  
Figure S8 Phylogenetic, sub-localization and expression analysis of two homologues of BnaLEC1.  
Figure S9 Expression of FA biosynthesis genes in bnalec1, BnaLEC1-ov and WT.  
Figure S10 Association of SNP polymorphisms with SOC across the BnaA6.RGA, BnaC7.RGA and BnaA7.LEC1.  
Figure S11 The expression patterns of GA biosynthesis genes during the seed development.  
Figure S12 The relative expression levels of FA biosynthesis genes after GA treatment.  
Table S1 DEGs between bnaa6.rga-D and WT in 100DAP and 200DAP of seed development stages determined by RNA-seq.  
Table S2 Expression levels of genes involved in TAG synthesis and assembly pathway in bnaa6.rga-D and WT in 100DAP and 200DAP of seed development stages determined by RNA-seq.  
Table S3 Expression levels of GDSL-type genes in bnaa6.rga-D and WT in 100DAP and 200DAP of seed development stages determined by RNA-seq. DEGs were marked in yellow.  
Table S4 The list of primers used for making constructs in this research.  
Table S5 The list of primers used for qRT-PCR expression analyses.