



Article

A High-Thousand-Seed-Weight Mutant of *Brassica napus*

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Abstract

Thousand-seed weight (TSW) is a critical determinant of yield in rapeseed (*Brassica napus* L.). Developing germplasm with high TSW is therefore a key strategy in high-yield rapeseed breeding. However, the genetic and molecular mechanisms underlying TSW in rapeseed remain poorly understood. In our earlier work, we identified a mutant, designated GRG177, which exhibits a remarkably high TSW exceeding 7 g. To unravel the mechanisms driving this elevated TSW, we conducted a comprehensive analysis of GRG177, integrating morphological, genetic, developmental, anatomical, and physiological approaches. Compared with the control germplasm GRD328 (TSW $\approx$ 3.5 g), GRG177 displayed a significant increase in seed weight and seed volume, larger silique surface area, and higher yield per plant. However, it also showed a notable reduction in both silique number per plant and seed number per silique. Genetic analysis of a segregating population revealed that the high-TSW trait in GRG177 is governed by two pairs of dominant epistatic major genes plus polygenes. Endogenous hormone analysis revealed significantly higher zeatin riboside (ZR) content in the early stage of seed development in GRG177, whereas indole-3-acetic acid (IAA) and abscisic acid (ABA) levels were significantly up-regulated in the late stage of seed development. Anatomical observation using paraffin sections further confirmed that enhanced cell division activity in the early stage and improved cell expansion capacity in the later stage underpin the formation of high TSW. Furthermore, BSA-seq was utilized to map four TSW-related Quantitative Trait Loci (QTLs) and screen 13 candidate genes involved in IAA, ZR, and ABA signaling pathways. In conclusion, these findings provide novel insights into the regulatory mechanisms governing high-TSW formation in rapeseed and present valuable genetic resources for high-yield breeding.



Academic Editor: Maoteng Li

Received: 29 December 2025

Revised: 8 February 2026

Accepted: 9 February 2026

Published: 14 February 2026

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Keywords: *Brassica napus* L.; high seed weight; genetic resource; yield improvement

1. Introduction

Rapeseed (*Brassica napus* L.) is one of the oil crops with the widest cultivation range globally. The breeding of high-yield rapeseed varieties is a core strategy to cope with the continuous growth of global vegetable oil demand and ensure the supply of edible vegetable oil [1,2]. Thousand-seed weight (TSW) is a key agronomic trait constituting rapeseed yield formation [3,4]. Exploring the new germplasm of rapeseed with high TSW

and analyzing its genetic rules and molecular mechanisms are of great significance for the improvement of high-yield rapeseed varieties.

Previous studies have demonstrated that TSW in rapeseed is co-regulated by the embryo, endosperm, and maternal plant [5,6]. The embryo and endosperm directly affect TSW mainly through three pathways. First, enhanced cell division activity in the embryo and endosperm promotes cell proliferation, which, in turn, increases seed volume and TSW [7–9]. Second, improved cell expansion capacity can also directly increase cell volume, laying the foundation for higher TSW [10,11]. Third, the coordination between cell division and cell expansion in seeds can drive the dynamic changes in TSW [12,13]. In addition, the maternal plant indirectly regulates TSW through nutrient supply via siliques. The photosynthetic capacity of rapeseed leaves gradually declines during the maturity stage, and approximately 70% of photosynthates in the late stage come from siliques [14]. Changes in the photosynthetic area of siliques affect the accumulation of storage substances in seeds, thereby regulating TSW [15]. Such maternal effects on seed weight regulation have been verified in crops such as maize, wheat, and rice [16,17].

Numerous studies have demonstrated that plant hormones such as indole-3-acetic acid (IAA) [18,19], cytokinin [20–22], and abscisic acid (ABA) [23,24] play crucial roles in the seed development. They can also affect seed weight by regulating the seed development of crops such as peanuts [25] and rice [26]. Notably, IAA directly regulates seed weight primarily through promoting endosperm cell expansion [27], whereas cytokinin [28] and ABA [29] affect seed weight by regulating the seed cell division. Specifically, zeatin riboside (ZR) is the most important cytokinin in rapeseed [30]. Furthermore, high concentrations of ABA in the late seed-filling stage can inhibit plant senescence and extend the seed-filling period [31,32]. This process may enhance the accumulation of seed substances, thereby increasing seed weight. However, the regulatory mechanism of endogenous hormones on rapeseed TSW has not been fully elucidated yet.

The TSW is a typical complex quantitative trait controlled by Quantitative Trait Loci (QTLs). Currently, it has been extensively studied in crops such as rice [33], soybean [34], wheat [35], and maize [36]. In *Brassica napus* L., research on QTL mapping related to TSW has also made some progress, with QTLs identified on almost all chromosomes [37–41]. The major QTL intervals are mainly located on A03 [37,42,43], A09 [12,44], C05 [45], and A02 [6]. Some candidate genes have been identified within these intervals, including *BnaA03.TGA6* [37], *BnDA1* [45], and *BnaA09.ARF18* [12], which negatively regulate TSW through the ubiquitin pathway, affecting the proliferation of seed coat cells, and inhibiting the expression of auxin-responsive genes, respectively. Meanwhile, *BnaA9.CYP78A9* [44] and *BnaC01.CCT8* [41] positively regulate TSW by increasing auxin content. Additionally, *BnaA03G37960D* [45] and *BnaA02b.SCPL19* [6] can positively regulate TSW by affecting cell division and development signaling, as well as abscisic acid signaling pathways. Despite some progress in the study of TSW in *Brassica napus*, the genetic regulatory network and interaction patterns of related genes remain unclear. Meanwhile, breakthrough germplasm resources with high TSW are particularly scarce, and the TSW of high TSW materials used for QTL interval mapping is mostly between 3.11 and 6.62 g. Although the highest TSW reported in previous studies was around 7.16 g [5], this study has not yet conducted QTL mapping and candidate gene mining.

In a previous study (the research has not been published), our team screened a *Brassica napus* germplasm GRG177 with a high TSW exceeding 7 g. In this study, GRG177 and the low-TSW line GRD328 (with a TSW of approximately 3.5 g) were used as experimental materials to conduct a systematic comparative analysis of phenotypes, development, cytology, and physiology. The genetic model of the TSW trait was analyzed by constructing the population. BSA-seq (Bulked Segregant Analysis by Sequencing) technology was used to

map TSW-related QTLs, and candidate genes were screened by combining homologous gene function annotation. Finally, the regulatory mechanism of the high-TSW trait in GRG177 was clarified. The findings of this study provide a critical foundation for advancing the genetic theory of TSW in rapeseed and lay a germplasm basis for high-yield rapeseed breeding.

2. Results

2.1. Variation in Agronomic Traits of the Mutant

In terms of field performance, the plant morphology of the two lines showed significant differences (Figure 1A), and there were also notable variations in seed size (Figure 1B,C). The average TSW increased from 3.53 g in GRD328 to 7.46 g in GRG177 (Figure 1D), while the average silique number per plant decreased from 283.27 in GRD328 to 237.73 in GRG177 (Figure 1E), and the average seed number per silique reduced from 16.02 in GRD328 to 11.13 in GRG177 (Figure 1F).

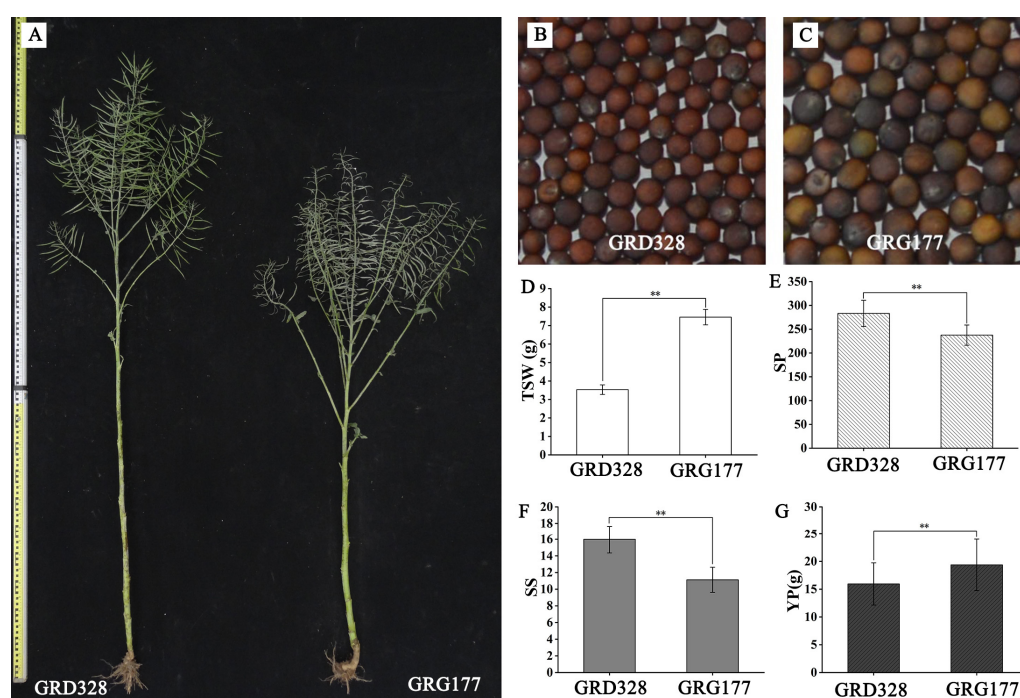


Figure 1. Agronomic characters of the mutant GRG177 and the control line GRD328. (A) Plant morphology of GRD328 (left) and GRG177 (right). (B) Seed photos of GRD328. (C) Seed photos of GRG177. (D) Comparison of thousand-seed weight (TSW). (E) Comparison of silique number per plant. (F) Comparison of seed number per silique. (G) Comparison of yield per plant. ** indicate significant difference level from results of *t*-test.

In addition, yield tests showed that the average yield per plant of the high TSW mutant was 19.39 g, which was higher than the 15.95 g of the control, with a yield increase range of approximately 10.62–28.2% (Figure 1G). This indicates that high TSW has the potential to improve rapeseed yield.

2.2. The TSW Trait of GRG177 Is Controlled by Two Pairs of Dominant Major Genes Plus Polygenes

To clarify the genetic basis of the high-grain-weight trait in GRG177, we constructed a six-generation population (P_1 , P_2 , F_1 , B_1 , B_2 , and F_2) using GRG177 and GRD328 as parents. The results showed that segregating generations (B_1 , B_2 , F_2) exhibited high genetic

polymorphism and a continuous distribution with multimodal patterns (Figure 2A–C), indicating that the TSW trait may be controlled by multiple major genes.

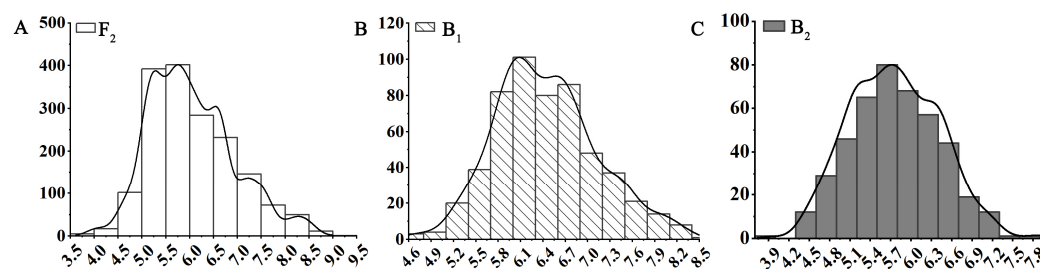


Figure 2. Frequency distribution of TSW in segregating generations. (A) Frequency distribution of TSW in F_2 generation. (B) Frequency distribution of TSW in B_1 generation. (C) Frequency distribution of TSW in B_2 generation. The y-axis represents frequency, and the x-axis represents TSW (g/1000 seed).

The goodness-of-fit test for TSW phenotypic data was performed using the major gene + polygene mixed genetic model for plant quantitative traits. The MX2-ADI-ADI model had the lowest Akaike Information Criterion (AIC) value and the fewest significant levels in the goodness-of-fit test (Table 1), which was the best genetic model for the TSW phenotype of the population. The model represented a mixed genetic model of two pairs of additive-dominant epistatic major genes + additive-dominant epistatic polygenes, suggesting that the TSW trait in this population is controlled by two pairs of major genes plus polygenes. Among them, the two pairs of major genes mainly exerted dominant effects, and high grain weight showed incomplete dominance over low grain weight. The values $|d_a| = |d_b|$ and $|h_a| < |h_b|$ indicated that the two pairs of major genes had the same additive effect, while the dominant effect of the second pair of major genes was more pronounced (Table 2). The interaction between the additive effects of the two pairs of major genes (i) was 0.62, the interaction between the dominant effects (l) was -0.15 , the additive $\times$ dominant interaction (j_{ab}) between the two pairs of genes was 0.17, and the dominant $\times$ additive interaction (j_{ba}) was 0.13, revealing obvious intergenic interactions. The heritability of the major genes in the segregating generations B_1 , B_2 , and F_2 was 54.74%, 26.24%, and 71.71%, respectively, while the heritability of the polygenes was 0.00%, 22.42%, and 4.37%, respectively.

Table 1. Maximum likelihood function MLV value and Akaike Information Criterion (AIC) value for segregation analysis of TSW optimal genetic model.

	Implication of Model ²	MLV ³	AIC ⁴	Test of Goodness-of-Fit ⁵
TSW ¹	MX2-ADI-ADI	−3210.77	6457.54	0/0/0/0/2
	MX1-AD-ADI	−3251.33	6526.67	0/0/1/2/3
	MX2-ADI-AD	−3271.42	6572.83	2/2/2/4/5
	PG-ADI	−3282.95	6585.90	1/1/0/1/2
	MX2-A-AD	−3292.00	6601.99	3/2/3/4/5
	2MG-ADI	−3318.31	6656.61	2/1/3/2/3
	MX1-NCD-AD	−3394.32	6804.65	4/5/4/6/6
	1MG-AD	−3399.00	6806.00	4/5/2/6/6
	PG-AD	−3403.94	6821.88	5/5/4/6/6
	MX2-EAD-AD	−3403.78	6823.56	5/6/3/6/6

¹ The 1000-seed weight. ² MX: mixed major gene and polygene model; PG: polygenic model; MG: major gene model; A: additive effect; D: dominance effect; I: interaction; N: negative; C: complete; E: equal; 1: one pair of major genes; 2: two pairs of major genes. ³ Max log likelihood value. ⁴ Akaike Information Criterion (AIC) value. ⁵ The five numbers denote numbers of significant statistic parameters of U_1^2 , U_2^2 , U_3^2 , ${}_nW^2$, and D_n . U_1^2 , U_2^2 and U_3^2 represent the goodness-of-fit tests based on mean, variance, and skewness, respectively. ${}_nW^2$ is the weighted variance statistic, and D_n is the skewness coefficient correlation statistic.

Table 2. Genetic parameter estimation of TSW.

Genetic Parameter *		1st Order										2nd Order				
		d_a	d_b	h_a	h_b	i	j_{ab}	j_{ba}	l	h_a/d_a	h_b/d_b	σ^2_p	σ^2_{mg}	σ^2_{pg}	h^2_{mg} (%)	h^2_{pg} (%)
Generation	B ₁											0.46	0.25	0.00	54.74	0.00
	B ₂	0.61	0.61	0.13	0.17	0.62	0.17	0.13	−0.15	0.21	0.28	0.4	0.11	0.09	26.24	22.42
	F ₂											0.87	0.62	0.04	71.71	4.37

* d_a , additive effect of the first major gene. d_b , additive effect of the second major gene. h_a , dominant effect of the first major gene. h_b , dominant effect of the second major gene. i , additive $\times$ additive interaction. j_{ab} , additive $\times$ dominance interaction. j_{ba} , dominance $\times$ additive interaction. l , dominance $\times$ dominance interaction. σ^2_p , phenotypic variance. σ^2_{mg} , major gene variance. σ^2_{pg} , polygenic variance. h^2_{mg} (%), major gene heritability. h^2_{pg} (%), polygenic heritability.

2.3. Variation in the Physiological Level of Mutant Seeds

In the early and middle stages of seed development, the seeds of GRG177 were significantly larger than those of the control (Figure 3A–E). However, there was no significant difference in seed size between the two lines at 41 days after flowering (DAF) (Figure 3F). Finally, the mutant again showed larger seeds from 44 DAF to 47 DAF (Figure 3G–H).

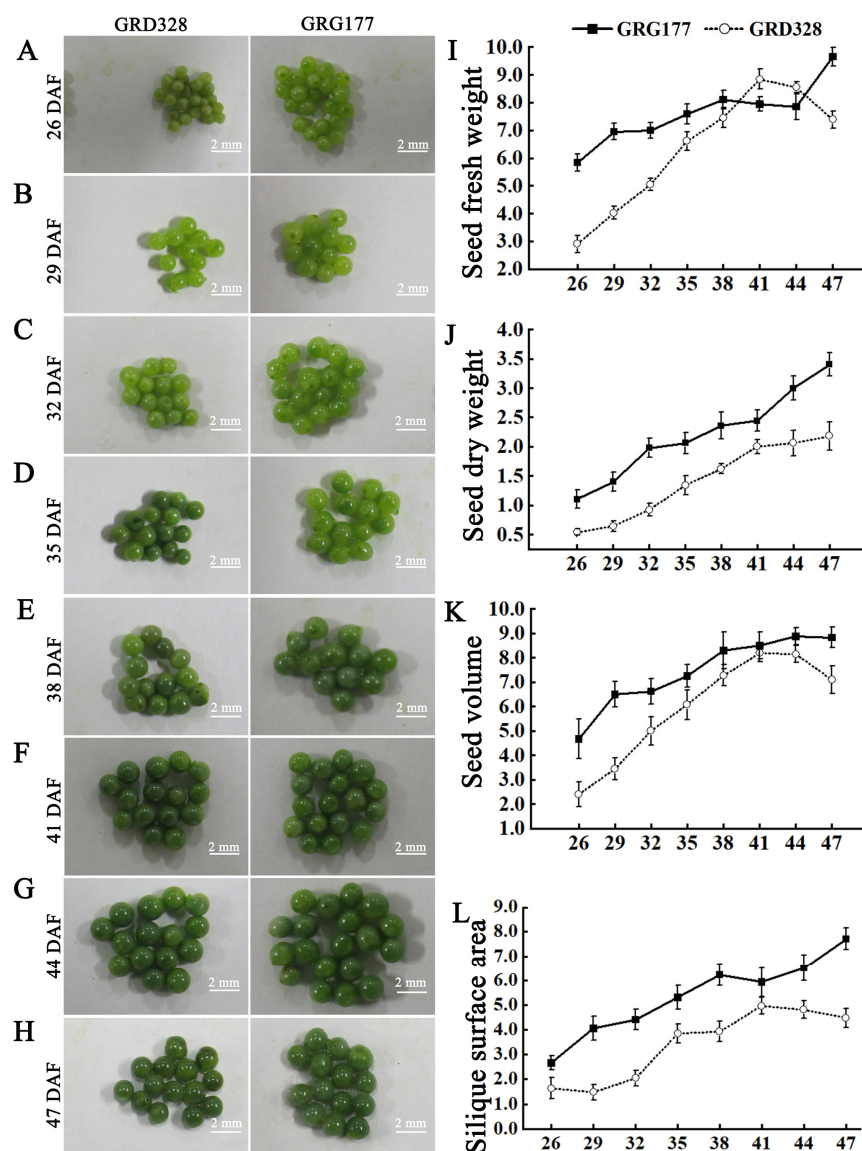


Figure 3. Seed differences between GRD328 and GRG177. (A–H) Comparison of dynamic development of seeds in two lines, scale bars = 2 mm. (I) The seed fresh weight (g/1000 seed). (J) The seed dry weight.

weight (g/1000 seed). (K) The seed volume (cm^3 /1000 seed). (L) The silique surface area (cm^2), and the abscissa indicates DAF.

During seed development, the average fresh weight (Figure 3I), the average dry weight (Figure 3J), and the average volume (Figure 3K) of GRG177 seeds were also significantly higher than those of GRD328, indicating distinct differences in the seed development process of the mutant. In addition, GRG177 had a larger-than-average silique surface area than the control during seed development (Figure 3L).

As the seeds developed, the seed dry weight, seed fresh weight, seed volume, and silique surface area increased continuously. However, the two lines showed obvious differences in the time when these indicators reached their peaks: all indicators of GRG177 peaked at 47 DAF, which was later than the peak time of GRD328 (41 DAF).

2.4. Variation in the Cells of Mutant Seeds

Based on the results that the mutant exhibited increased seed weight and volume, we hypothesized that there were differences in cells between the mutant and control seeds. Therefore, paraffin sectioning was used to observe cellular changes during seed development.

The results showed that, at 26 DAF, the average number of cells in GRG177 was significantly higher than in the control (Figure 4A). The average number of cells per unit area in GRG177 was 120 cells per unit area, which was much higher than the 60 cells per unit area in GRD328 (Figure S1). From 29 DAF to 38 DAF, there was no significant difference in the mean number and mean size of cells between the two lines (Figure 4B–E). However, after 41 DAF, the average cell volume of GRG177 was significantly larger than that of the control (Figure 4F), and, after 44 DAF, the magnitude of the increase in mean cell volume was higher in GRG177 than in GRD328 (Figure 4G–H).

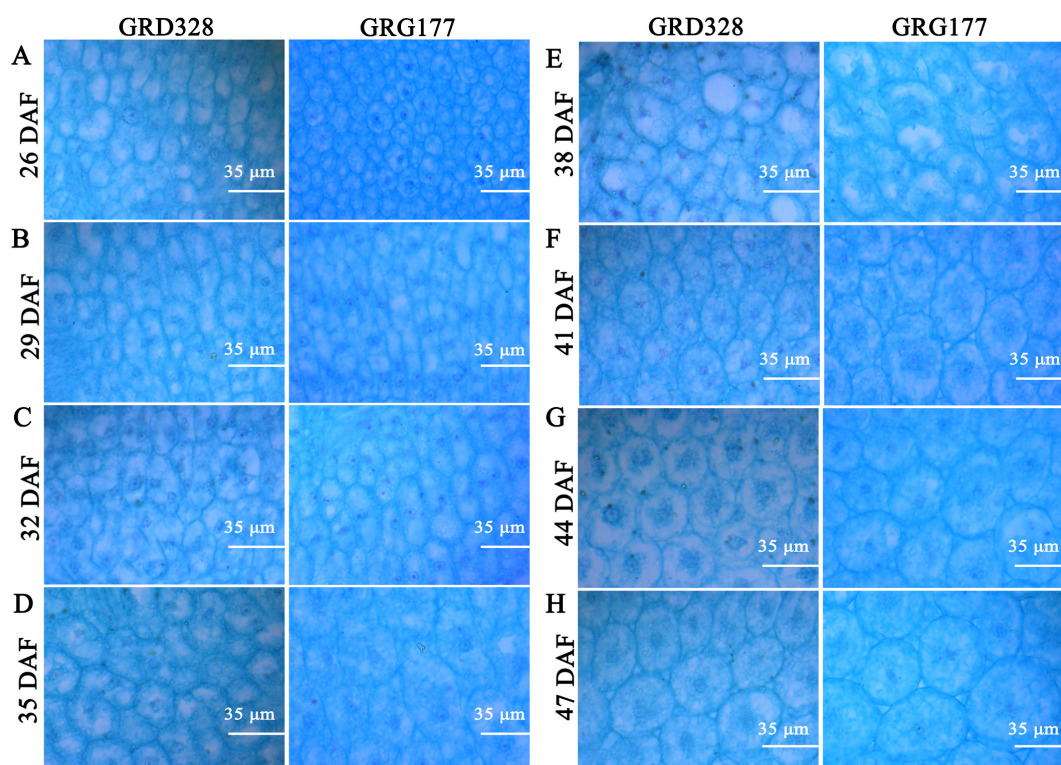


Figure 4. Comparison of seed paraffin sections of GRD328 (left) and GRG177 (right). (A–H) Comparison of dynamic development of seed cells in two lines, scale bars = 35 μm , and DAF represents DAF.

2.5. Variation in Endogenous Hormones of Mutant Seeds

Since GRG177 differs from GRD328 in both cell number and cell size, and plant hormones are key substances regulating plant cell proliferation and growth, we hypothesized that these differences are associated with changes in endogenous plant hormones. Therefore, we determined the contents of relevant endogenous hormones— indole-3-acetic acid (IAA), zeatin riboside (ZR), and abscisic acid (ABA)—in the seeds of the two lines. The results showed that the IAA content of GRG177 was significantly lower than that of GRD328 in the early stage of seed development; then, it gradually increased in the middle and late stages and became higher than that of GRD328 after 41 DAF. At 32 DAF, the IAA content of GRD328 reached its peak at 3482.3171 ng/g, which was five times that of GRG177. At 44 DAF, the IAA content of GRD328 dropped to its lowest level (734.23 ng/g), approximately 50% of that of GRG177 (Figure 5A). The ZR content of GRG177 was extremely significantly higher than that of GRD328 at 26 DAF, lower than that of GRD328 at 29 DAF, slightly higher than that of GRD328 from 32 DAF to 35 DAF, and showed no obvious difference afterward (Figure 5B). The ABA content of both lines fluctuated greatly, with an overall trend of first decreasing and then increasing. However, the ABA content of GRG177 was generally higher than that of GRD328 (Figure 5C).

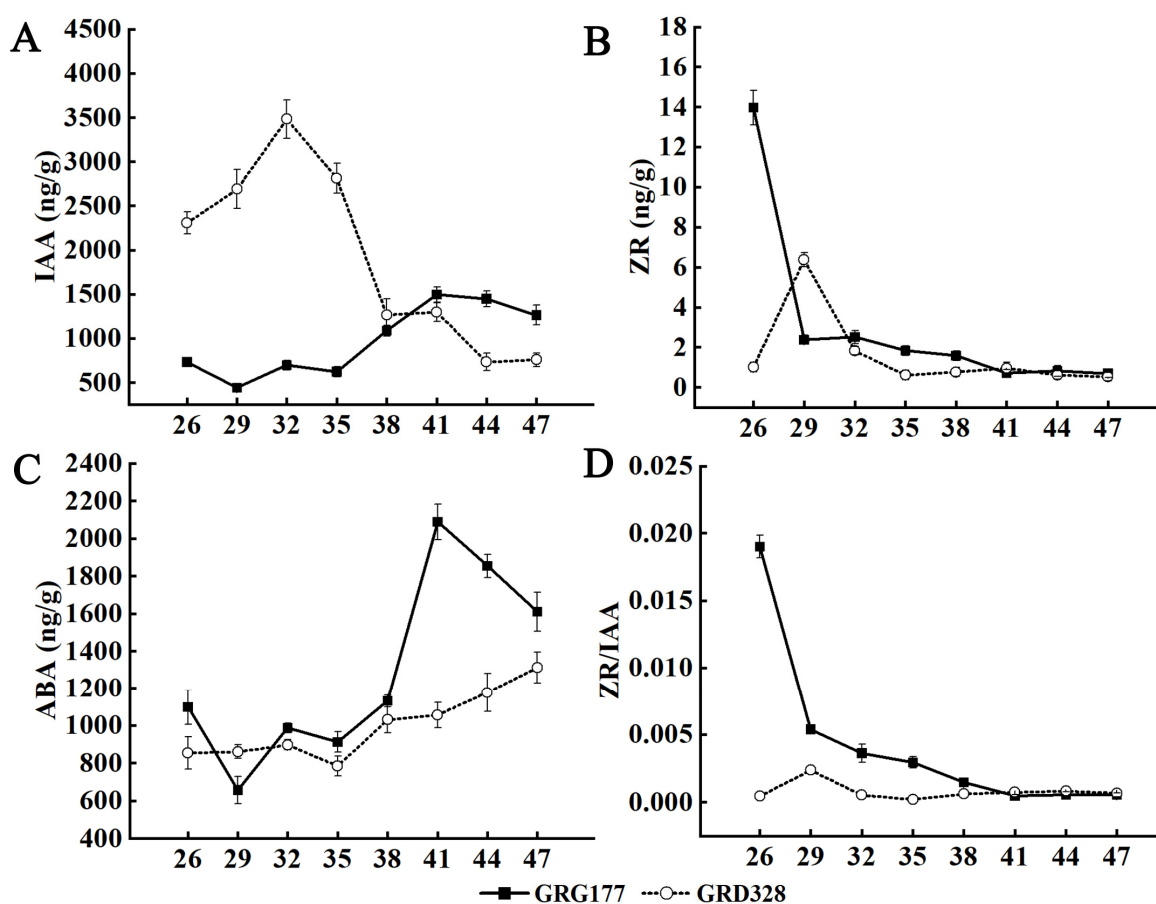


Figure 5. Comparison of endogenous hormone content in GRD328 and GRG177 seeds. (A) Dynamic developmental comparison of Indole-3-acetic acid (IAA) content. (B) Dynamic developmental comparison of zeatin riboside (ZR) content. (C) Dynamic developmental comparison of abscisic acid (ABA) content. (D) Dynamic developmental comparison of ZR/ IAA ratio, and the abscissa indicates DAF.

Both IAA and ZR are important hormones involved in plant growth and development. However, the results of this study showed that the change in trends of IAA and ZR content

were opposite in the two lines. Further investigation revealed that the high-grain-weight mutant had a higher ZR/IAA ratio. At 26 DAF, there was an extremely significant difference in ZR/IAA between the two lines: the ZR/IAA of GRG177 (0.0190) was 38.5 times that of GRD328 (0.0004). From 29 DAF to 38 DAF, the gap between the two lines gradually narrowed, but the ZR/IAA of GRG177 was still significantly higher than that of GRD328. After 41 DAF, the ZR/IAA of the mutant was slightly lower than that of the control (Figure 5D).

2.6. QTL Mapping for the TSW Trait

To unravel the regulatory mechanism underlying the high-TSW trait in rapeseed (*Brassica napus* L.), we performed whole-genome resequencing on two parental lines and two extreme trait pools from the F₂ population, and the screening threshold of extreme characters was 5%. We detected SNPs (Single Nucleotide Polymorphisms) and InDels (Insertions and Deletions) with BSA (Bulked Segregant Analysis) mapping. By screening polymorphic loci between the parents, we identified a total of 3,687,337 SNPs and 919,850 InDels. To narrow down the scope, the 95% confidence level was set as the screening threshold, leading to the identification of four QTLs (Quantitative Trait Loci) on chromosomes A03, A08, C02, and C06 (Figure 6A–D). Among these QTLs, the largest interval was located at 27.51–29.74 Mb on chromosome A03, spanning 1.141 Mb and containing 67 genes. All QTLs collectively covered a total length of 2.098 Mb and included 189 genes.

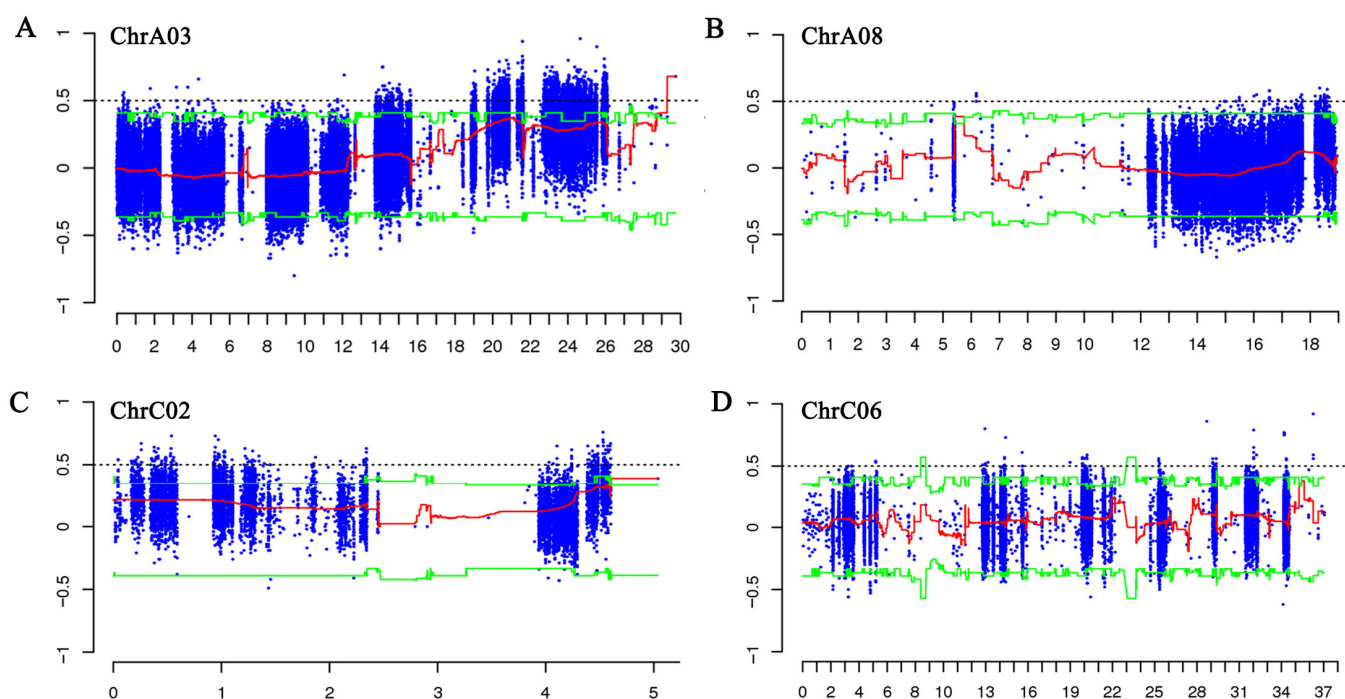


Figure 6. QTL intervals located on chromosomes. (A) This figure illustrates the distribution of $\Delta(\text{All-index})$ on chromosome A03. (B) This figure illustrates the distribution of $\Delta(\text{All-index})$ on chromosome A08. (C) This figure illustrates the distribution of $\Delta(\text{All-index})$ on chromosome C02. (D) This figure illustrates the distribution of $\Delta(\text{All-index})$ on chromosome C06. With chromosome length (Mb) on the x-axis and $\Delta(\text{Index})$ on the y-axis. Loci above the threshold, where the red curve exceeds the green threshold line, are defined as candidate intervals.

2.7. Screening of Candidate Genes for the TSW Trait

Based on the BSA-seq (Bulked Segregant Analysis sequencing) result analysis, there were 60 SNP variant loci and 59 InDel variant loci in the candidate intervals. We performed

functional annotation of genes harboring reference SNP and Indel variant sites based on the reference genome. By searching for their homologous genes against the COG (Clusters of Orthologous Groups) database, we screened genes with functional annotations related to hormones or cell growth as candidate genes. The results showed that there were 13 potential candidate genes associated with TSW on four chromosomes (Table S1). Among these candidate genes, six were related to ABA, two to ZR, four to IAA, one to ubiquitin, and two to cell elongation.

We analyzed the expression sites and levels of these candidate genes in *Brassica napus* using the BnIR (*Brassica napus* Information Resource) database and found that all candidate genes reached their highest expression levels in seeds and tissues related to seed development, such as siliques and anthers.

3. Discussion

TSW (thousand-seed weight) is one of the three key factors determining rapeseed yield per plant, and increasing TSW is an important approach to enhance the yield potential of rapeseed varieties. Although multiple QTLs (Quantitative Trait Loci) associated with TSW in *Brassica napus* have been mapped in previous studies, the TSW of germplasm materials used for genetic research is generally limited to below 6.0 g. This has greatly restricted the elucidation of the genetic architecture underlying the TSW trait in rapeseed.

In this study, we analyzed a previously identified high-TSW *Brassica napus* mutant (the research has not been published), GRG177 (with a TSW exceeding 7 g). Compared with the control line, GRG177 exhibited fewer siliques per plant and fewer seeds per silique, but a significantly higher TSW and yield per plant. This finding differs from the conclusions of previous studies [3], which reported that high-TSW materials had reduced yield per plant. The reason for this discrepancy may be that the TSW of plants used in previous studies was relatively low, with a maximum of approximately 6.6 g [22]. In contrast, the TSW of the mutant used in this study was much higher than that of these materials. This substantial increase in TSW offsets the reductions in the number of siliques per plant and seeds per silique, resulting in a higher yield per plant for GRG177. These results indicate that high-TSW mutants have great potential for improving rapeseed yield. In addition, the silique surface area of the mutant was significantly larger than that of the control. As siliques serve as the main photosynthetic organs during the late developmental stage of rapeseed [14], the increased silique surface area is more conducive to the accumulation of photosynthates, which, in turn, promote the formation of high TSW. The multiple differences observed in the mutant led us to hypothesize that the TSW trait is regulated by multiple genes. This inference was verified by constructing a segregating population: the TSW trait of the mutant is controlled by a mixed genetic model consisting of two pairs of dominant major genes plus polygenes.

We further conducted dynamic observations on the seed development process of the mutant and found that the dry weight, fresh weight, and volume of GRG177 seeds were all significantly higher than those of the control. However, as the seeds developed, the seed volume of both lines did not increase continuously; instead, the volume of both lines decreased after 41 DAF. Previous studies have shown that rapeseed seed volume is positively correlated with water content [46,47]. In the late stage of seed maturation (approximately after 40 DAF), water is lost from the intercellular spaces and vacuoles, leading to cell shrinkage and a reduction in seed volume. Moreover, after this stage, the seed size becomes basically fixed [48,49]. The decrease in seed volume of GRG177 was significantly smaller than that of GRD328, which may also be one of the reasons for the higher seed weight of the mutant.

In addition, the results of paraffin sections on the seed cell development process showed that GRG177 had a greater number of cells in the early stage of seed development and larger cell volume in the late stage. Based on these findings, we further determined the contents of hormones related to cell division and growth during seed development. The results revealed that the zeatin riboside (ZR) content of the mutant at 26 days after flowering (DAF) was much higher than that of the control. Previous reports have also found that rice and wheat have excessively high ZR content when endosperm cell division is the most vigorous [50], which is consistent with the result of increased cell number obtained from paraffin sections. The control plants had higher indole-3-acetic acid (IAA) content in the early stage; after 41 DAF, their IAA content began to decrease and became lower than that of GRG177. At 44 DAF, the IAA content of the control reached the minimum, while the cell volume reached the maximum. In contrast, GRG177 maintained a relatively high IAA content after 44 DAF, and the cell volume observed in paraffin sections continued to increase. This suggests that the higher IAA content of GRG177 in the late stage of seed development may promote continued cell growth, thereby increasing the TSW. Studies have shown that high concentrations of ABA are associated with cell number [29] and can inhibit plant senescence during the late grain-filling stage [31,32]. Compared with the control, GRG177 had higher abscisic acid (ABA) content in the late developmental stage, which may be involved in regulating the extension of the seed development cycle to promote the formation of high TSW.

Furthermore, the interaction between different hormones can also affect the seed development process. For example, the interaction between IAA and ABA influences seed development in rice [51], alfalfa [52], and licorice [53]. The ratio of ZR to ABA is also related to embryonic development [54]. Therefore, we hypothesized that changes in the ratios between these hormones are also associated with changes in TSW. Through comparison, we finally found that the high-grain-weight mutant had a higher ZR/IAA ratio in the early stage of seed development, which was up to 38.5 times that of the control.

A study has shown that the number of cells in rapeseed seeds has a greater impact on TSW than cell size [7]. Combined with the development of seed cells, it can be concluded that the higher ZR/IAA ratio in the early stage makes the cells of the mutant seeds more inclined to undergo cell division rather than an increase in volume. This results in a significant increase in the number of cells in the seeds, thereby increasing the TSW. In the late developmental stage, the decrease in the ZR/IAA ratio causes the nutrient supply of the seeds to be mainly consumed in seed growth and development rather than proliferation. The changes in seed cells we observed also verified this result.

To unravel the regulatory mechanism underlying high-TSW formation, we identified four QTLs associated with the TSW trait via BSA-seq. Among these QTLs, one highly overlaps with the TSW-related QTL previously mapped on chromosome A08 by Fan et al. [39], while the other three are novel TSW-associated QTLs. These QTLs may contain new genes that regulate TSW.

Thus, we further screened the genes within these intervals and finally selected 13 TSW-related candidate genes. Among them, the gene *BnaA03g54360D*, which is related to the E3 ubiquitin-protein ligase EDA40, may affect TSW by regulating the timing of cell division [55]. The candidate gene *BnaC02G48490D* is annotated as elongation factor 1-alpha, which may be involved in seed elongation. The actin-binding FH2 family protein-related gene *BnaA03g54550D* may influence cell division by regulating processes such as cell assembly and polymerization [56]. All the aforementioned genes are consistent with the results observed in this study via paraffin sections. In addition, among the genes related to hormone regulation, three genes—*BnaC02g48550D*, *BnaA08g05710D*, and *BnaA08g05580D*—are involved in IAA synthesis [51,57,58]. The candidate gene

BnaC06G37330D was annotated as a membrane transport protein, and its homologue in *Arabidopsis thaliana*, *AT1G70940*, was annotated as an auxin efflux carrier family protein, which is involved in the development of *Arabidopsis* embryos. The expression of the target gene in *Arabidopsis* embryos was analyzed [59,60]; therefore, it is also possible that this candidate gene is related to the development of seed embryos in *Brassica napus*. The candidate gene *BnaA03g54500D* may regulate ABA release [61], and *BnaA03g54390D*, *BnaA03g52920D*, and *BnaA03g54350D* may participate in ABA signal transduction [62,63]. The candidate genes *BnaA03g52860D* and *BnaA08g05630D* may be related to ABA metabolism [64,65], ZR degradation [66], and ZR synthesis [67]. Given the significant changes in these hormones observed in the mutant, the aforementioned genes are potential candidate genes associated with TSW.

4. Materials and Methods

4.1. Plant Materials

The high-TSW (thousand-seed weight) mutant *Brassica napus* line GRG177 (TSW ~7.5 g) and the conventional line GRD328 (TSW ~3.5 g) were used as the experimental and control materials, respectively. GRG177 is a high-TSW semi-winter rapeseed (*Brassica napus* L.) natural mutant found in self-bred lines, and GRD328 is a conventional low-TSW semi-winter rapeseed line. F₁ plants were obtained by crossing GRG177 and GRD328 in 2018. In 2019, a segregating population F₂ was obtained via the selfing of F₁ plants, and B₁GRG177 (B₁) and B₁GRD328 (B₂) were obtained by backcrossing F₁ plants with GRG177 and GRD328, respectively. These crosses constituted a genetic population (P₁, P₂, F₁, F₂, B₁, and B₂). All plant materials were provided by the Guizhou Rapeseed Research Institute (Guiyang, China) and cultivated under natural field conditions at their experimental base.

4.2. Field Trials and Phenotyping

The field experiment was conducted in 2020 at the experimental base in Weiyuan Town, Guizhou Province, China (26°01'36" N, 106°31'43" E, altitude 970 m), within a rice-rapeseed rotation field. All materials were uniformly raised in a nursery. At the seedling stage, plants were randomly transplanted with a spacing of 40 cm between rows and 25 cm between plants, and each row contained 12 plants [68]. P₁ and P₂ were each transplanted with 3 rows, F₁ with 10 rows, F₂ with 150 rows, and B₁ and B₂ were each transplanted with 75 rows, divided into 3 replicates. All the plants were numbered, and some young leaves were taken at seedling stage (3–5 leaf stage) and stored in self-sealing bags in a −80 °C refrigerator [40]. Before transplantation, a compound fertilizer (N:P:K = 1:1:1) was applied as base fertilizer at a rate of 375 kg·ha^{−1}, and urea was used as topdressing at a rate of 75 kg·ha^{−1}. Field irrigation and management followed standard agricultural practices.

At the initial flowering stage, plants with uniform growth vigor and initial flowering time were marked for subsequent trait determination and sampling. From 26 days after flowering (DAF), 50 siliques of each generation were randomly sampled, and the growth state of the samples was ensured to be consistent, and the data measurement was completed within 2 h after sampling. Samples were taken every 3 days until the seeds were close to developmental maturity, for a total of 8 times. After sampling, the length and width of silique body and beak were measured, respectively. The sum of fruit body length and beak length was used as silique length, and the sum of fruit body width and beak width was used as silique width, and silique surface area (s) was calculated with reference to Clarke and Simpson [69].

$$S = \pi dh1 + \frac{1}{3}\pi dh2$$

$h_1 = 0.8 H$ and $h_2 = 0.2 H$, where H represents the silique length and d represents the silique width.

Then, the fresh weight of seeds in a single silique using an analytical balance was weighed; due to the small size of the seeds, the average value was taken and converted to the fresh weight per thousand seeds. Photographs were taken at the same level to record and compare seed size. Meanwhile, the seed volume was measured using the water displacement method, and the average value was taken and converted to the volume per thousand seeds. Finally, the dry weight of the seeds was measured after drying and converted to the dry weight per thousand seeds, and part of the fresh sample from each plant was stored in a $-80\text{ }^{\circ}\text{C}$ refrigerator. At the maturity stage, the number of siliques per plant and the number of seeds per silique were investigated, and the TSW was measured after seed harvesting and drying.

4.3. Paraffin Embedding and Sectioning

The method was adapted from Cao et al. [70] with minor modifications. Seeds of each period previously sampled were immediately placed in precooled FAA (formalin–acetic acid–alcohol) fixative ($4\text{ }^{\circ}\text{C}$) after being removed from an ultra-low-temperature refrigerator at $-80\text{ }^{\circ}\text{C}$, followed by graded dehydration in 70%, 85%, 95%, and 100% ethanol (2–4 h per step). The samples were then subjected to cyclic clearing using a mixture (50% xylene and 50% ethanol, v/v) and pure xylene. After that, the materials underwent wax infiltration, embedding, sectioning, and dewaxing. Finally, the sections were stained sequentially with safranin and fast green solution, followed by a drying step. After drying, each section was separately observed and analyzed under an Olympus inverted microscope (model: CKX41SF, Olympus Corporation, Tokyo, Japan). Images were taken using the microscope's built-in high-resolution digital camera. The experiment was repeated 3 times.

4.4. Endogenous Hormone Measurement

First, approximately 0.2 g of previously collected seeds from each period were immediately frozen in liquid nitrogen and ground to a fine powder. The powder was thoroughly mixed with 1 mL of 80% (v/v) methanol and sonicated in an ultrasonic bath, and then stored at $4\text{ }^{\circ}\text{C}$ overnight. Following centrifugation at $15,200\times g$ for 10 min, the supernatant was collected and concentrated by vacuum centrifugation to remove methanol. Subsequently, 200 μL of 0.1 mol/L sodium phosphate buffer (pH 7.8) was added to the residue. The mixture was loaded onto a Sep-Pak C18 solid-phase extraction cartridge and eluted with 1.5 mL of 80% (v/v) methanol. The eluate was dried under vacuum and reconstituted in 10 μL of 10% (v/v) methanol. Finally, 5 μL of the reconstituted solution was injected into a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system (8030 Plus, Shimadzu Corporation, Kyoto, Japan) for hormone quantification, following the protocol described by Tang et al. [68]. The experiment was repeated 3 times.

4.5. Genetic Analysis

Genetic analysis was performed using the major gene plus polygene mixed inheritance model data package [71] of R language software (software version 4.5.1). Candidate models were selected based on the Akaike Information Criterion (AIC), and the best-fitting model was identified through goodness-of-fit tests, including homogeneity tests (U_1^2 , U_2^2 , U_3^2), Smirnov test (${}_nW^2$), and Kolmogorov test (D_n). Maximum likelihood method and Iterative Expectation and Conditional Maximization (IECM) algorithm were employed for parameter estimation of each generation. The dominance effect, additive effect and gene interaction of two pairs of major genes were evaluated by genetic parameters in the best-fit genetic model, and the heritability of major genes and polygenes was calculated. The heritability of major genes was calculated as $h_{mg}^2 = \sigma_{mg}^2 / \sigma_p^2$ and the heritability of polygenes as

$h^2_{pg} = \sigma^2_{pg} / \sigma^2_p$. Here, σ^2_p , σ^2_{mg} , and σ^2_{pg} represent the total phenotypic variance, genetic variance of major genes, and genetic variance of polygenes, respectively.

4.6. Library Construction and Sequencing

Based on phenotypic data from the F₂ population, 20 individuals with the highest TSW and 20 with the lowest TSW were selected. Corresponding leaf samples, previously stored at −80 °C, were retrieved according to their identifiers. Genomic DNA was extracted from a total of 42 samples, including the 40 selected F₂ plants and the two parental lines (GRG177 and GRD328). DNA purity and integrity were assessed by agarose gel electrophoresis. The OD₂₆₀/OD₂₈₀ ratio was measured using a spectrophotometer (NanoDrop Lite Plus spectrophotometer, Thermo Scientific™, Waltham, MA, USA), and DNA concentration was precisely quantified with a fluorometer (Invitrogen Qubit fluorometer, Thermo Scientific™, Waltham, MA, USA). High-quality DNA samples were randomly fragmented to an average size of 350 bp using a sonicator (Covaris-LE220R, Thermo Scientific™, Waltham, MA, USA). Libraries were prepared using the DNA Library Prep Kit (Illumina TruSeq DNA Library Prep Kit, Illumina, Inc., San Diego, CA, USA), strictly adhering to the manufacturer's protocol. The workflow included end repair, 3'-A tailing, adapter ligation, size selection, and PCR enrichment to complete library construction. Libraries constructed according to the above procedure were combined after concentration homogenization, SBS double-ended sequencing was performed on Illumina sequencer (Illumina HiSeq™ PE150, HiSeq 2500, Illumina, Inc., San Diego, CA, USA) into base sequences, and, finally, FASTQ raw data were generated.

Whole-genome resequencing was further performed on two parental lines and two extreme trait pools in an F₂ population with a screening threshold of 5% for extreme traits. SNPs (Single Nucleotide Polymorphisms) and InDels (Insertions and Deletions) were detected by BSA (Bulked Segregant Analysis). The alignment rates for all samples ranged from 98.62% to 98.66%, with an average sequencing depth of 27.62× to 38.55×.

4.7. QTL Mapping and Candidate Gene Screening

The constructed library was sequenced (Illumina HiSeq 2500, Illumina, Inc., San Diego, CA, USA), followed by alignment and statistical analysis against the *Brassica napus* reference genome (Darmor-bzh, genome size: 850,292,103 bp). The UnifiedGenotyper module of GATK 3.8 software was used to detect SNPs and InDels across multiple samples [72]. One parent was selected as a reference, and BMKCloud (Baimaike Biotechnology Co., Ltd., Wuhan, China) was used to calculate the allele frequency difference between the two extreme mixed pools, and the SNP index and Indel Index of polymorphic marker loci between the two offspring populations were analyzed. Loci with values less than 0.3 in both pools were filtered out. To intuitively reflect the chromosomal distribution of the combined All-index (merged from the offspring's SNP-index and InDel-index), a plot was generated to visualize the distribution of All-index across chromosomes. The Δ(All-index) was calculated using BMKCloud and 1000 permutation tests were performed. A 95% confidence level was set as the screening threshold, and windows with values exceeding this threshold were designated as candidate intervals. To avoid neglecting the effects of minor-effect QTLs, candidate SNPs and InDels were selected across the whole genome, laying the foundation for the subsequent mining of minor-effect QTLs. ANNOVAR software (software version 2013Aug23) was used to annotate SNP/InDel mutations and predict the impact of these mutations on gene function [73].

For these mutated genes, we integrated gene annotations from the reference genome and searched for their homologous genes through the COG (Clusters of Orthologous

Groups, <https://www.ncbi.nlm.nih.gov/research/COG>, accessed on 17 August 2025) database. By referencing homologous genes with verified function, we screened out candidate genes related to seed development and other related processes. Finally, comprehensive tissue expression analysis of these candidate genes was conducted using the BnIR (*Brassica napus* Information Resource, <https://yanglab.hzau.edu.cn/BnIR>, accessed on 28 August 2025) database to validate the expression of these candidate genes.

5. Conclusions

In this study, we performed a comprehensive analysis of the high-TSW (thousand-seed weight) mutant GRG177, covering aspects including agronomic traits; genetic inheritance; physiological, cellular, and hormonal changes during seed development; and gene expression. This analysis clarified the intrinsic connections among variations across multiple levels and established a regulatory network underlying its TSW trait. The core mechanism involves mutations in multiple endogenous hormone-associated genes, which synergistically enhance cell proliferation during the early stages of seed development and cell expansion during the later stages, ultimately contributing to the high-TSW phenotype. These findings not only provide key germplasm resources and technical support for breeding high-TSW rapeseed germplasm and high-yield varieties, but also serve as valuable references for the fine mapping, cloning, and functional analysis of TSW-related genes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041852/s1>.

Author Contributions: Y.X. designed the research, analyzed the data and wrote the paper. Z.F. analyzed the data and wrote the paper. X.L. performed the field experiments and analyzed the data. Y.Z. (Yifei Zou) analyzed the data and wrote the paper. L.L., R.L., Y.Z. (Yan Zhang), W.L. and H.H. performed the field experiments. L.X. and J.T. performed hormone measurement experiments. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Scientific and Technological Key Program of Guizhou province (No. Qiankehezhicheng [2022] Key 031), the National Natural Science Foundation of China (32070217) subsidy project from NSFC of Guizhou Academy of Agricultural Sciences (No. [2021] 50) and Construction of Innovation Capacity for the Basic Platform of Breeding Research in Guizhou Province (Qiankehefuqi [2022] No. 014).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Acknowledgments: We would like to thank Novogene for helping with the BSA-seq work.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TSW	Thousand-seed weight
SP	Silique number per plant
SS	Seed number per silique
YP	Yield per plant
DAF	Days after flowering
IAA	Indole-3-acetic acid
ZR	Zeatin riboside

ABA Absciscic acid

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