

Development of molecular markers associated with saline-alkali tolerance in rapeseed (*Brassica napus* L.)

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**Development of molecular markers associated with saline-alkali
tolerance in rapeseed (*Brassica napus* L.)**

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Abstract

Key message

Candidate gene-based association analysis identified 483 saline-alkaline tolerance-associated genes and enabled the development of 947 KASP and InDel markers in rapeseed. These markers provide a foundation for allele tracking and for integrating saline-alkaline tolerance into molecular design breeding systems.

Abstract: Salt-tolerant rapeseed (*Brassica napus* L.) plays a pivotal role in the sustainable utilization and improvement of saline-alkaline soils. However, current breeding of highly tolerant cultivars still relies largely on conventional hybridization combined with phenotype-based selection, which is strongly influenced by environmental variation, labor-intensive, and low-throughput, thereby limiting breeding efficiency. To address these challenges, a total of 1,281 candidate genes previously reported across rice, maize, wheat, sorghum, and *Arabidopsis* were compiled. Subsequent candidate gene-based association analysis identified 483 significantly associated genes, 355 of which harbored favorable haplotypes. Molecular markers were efficiently developed for 275 genes, including 746 KASP and 201 InDel marker pairs. Four marker pairs were randomly selected for validation, and the results demonstrated their effectiveness in classifying germplasm based on tolerance levels. Given the relatively small effect of individual saline-alkaline tolerance loci in rapeseed, these markers enable efficient pyramiding of favorable alleles into parental lines, thereby accelerating the development of highly tolerant varieties. This study provides essential tools for early-generation evaluation and marker-assisted selection (MAS) and lays a solid foundation for the molecular design breeding of saline-alkaline-tolerant rapeseed

Introduction

Soil salinization has become a major global concern. According to the Food and Agriculture Organization of the United Nations (FAO), approximately 1.4 billion hectares of land worldwide are affected by salinity, accounting for about 10.7% of the global terrestrial area. Moreover, salinity impacts roughly 10% of both irrigated and rainfed croplands. This situation underscores the urgent need to establish effective

reclamation strategies to promote the sustainable utilization of arable land. Traditional remediation approaches generally combine physical, chemical, and biological measures to restore soil productivity (Hu et al. 2022; Pei and Yu 2023; Yao et al. 2023). While these methods have to some extent improved soil quality and increased crop yields, large-scale restoration of saline-alkali soils remain challenging, mainly due to high costs and considerable ecological risks associated with such practices (Du 2024).

Among the three principal approaches for improving saline-alkali soils, biological amelioration is widely recognized as a sustainable and effective strategy. Rapeseed (*Brassica napus* L.), a vital oilseed crop with considerable economic and nutritional value, also demonstrates strong potential in saline-alkali soil remediation owing to its stress tolerance and soil-restoring capacity (Wang et al. 2021). For example, the cultivar ‘Huayouza 7’ yielded over 1950 kg/ha in soils with salinity levels of 0.6 – 0.8‰, while ‘Huayouza 62’ tolerated NaCl levels of 1.2‰ and up to 1.5‰ under pot conditions (Wang et al. 2021). Rapeseed shoots accumulated Na⁺ at a concentration of 5.28 mg/g, which is 15.5 times and 13.9 times higher than that in wheat (0.34 mg/g) and soybean (0.38 mg/g), respectively. Following rapeseed cultivation, soil Na⁺ content decreased by 46.4% compared to control plots and other crops (Wang et al. 2022). Furthermore, in field trials conducted in Jilin Province, rapeseed cultivation reduced soil pH from 8.48 to 8.26 and salinity from 0.41‰ to 0.375‰, while significantly enhancing soil fertility. Specifically, organic matter increased by 29.40%, total nitrogen by 25.23%, available phosphorus by 74.18%, and potassium by 86.17 % (Wang et al. 2021). These results collectively underscore the importance of breeding saline-alkali-tolerant rapeseed varieties to facilitate the transformation of saline-alkali soils into productive resources, ensure oilseed supply, and promote sustainable agricultural ecosystems.

With rapid advances in genomics, multi-omics technologies, and AI-assisted prediction, molecular design breeding has become a key strategy for rapeseed improvement (Hickey et al. 2019; Song et al. 2020; Li et al. 2023, 2025). The availability of high-quality reference genomes, pangenomes, large resequencing cohorts, and multi-environment GWAS datasets has greatly expanded the genetic

resources for Brassica research(Yang et al. 2023). These data now allow more precise dissection of gene networks and favorable allelic variants underlying major agronomic traits(Zhang et al. 2025), thereby improving genotype-based prediction and enabling the targeted assembly of superior allele combinations(Tong et al. 2025).

Within this breeding framework, molecular marker – assisted selection (MAS) provides an essential bridge between genomic discovery and breeding practice. It enables breeders to efficiently track causal genes, favorable haplotypes, and elite multi-locus allele combinations. In crop salt-tolerance research, markers linked to major loci such as SKC1/HKT1(Ren et al. 2005), OsHAL3(Xiang et al. 2022), as well as numerous QTLs identified across multiple species (Zhou et al. 2016; Luo et al. 2021; Mondal et al. 2022)have been successfully deployed for trait improvement. In rapeseed, genomic studies have revealed extensive genetic variation for salt tolerance, including 62 QTLs related to shoot biomass and ion homeostasis (Yong et al. 2015), 75 QTLs associated with seed germination under salt stress, and nearly 3,000 SNPs significantly correlated with tolerance (Wan et al. 2018; Zhang et al. 2021). More recently, two SNP markers co-segregating with salt tolerance were reported(Yang et al. 2024).

Despite the substantial progress in identifying QTLs, SNP markers, and candidate genes associated with salt tolerance in rapeseed, their translation into breeding practice remains limited. This gap largely reflects the complex genetic architecture of salt and alkali tolerance, which is typically controlled by numerous small-effect loci and shaped by strong genotype – environment interactions(Cheng et al. 2025). In addition, stable and breeder-friendly marker systems are still lacking, and practical strategies for assembling multi-locus favorable alleles have not been fully established. Therefore, clarifying the multilocus genetic basis of tolerance and developing reliable markers that support allele pyramiding and early-generation selection are essential steps for advancing rapeseed improvement toward a true molecular design breeding paradigm.

Given these challenges, the development of effective molecular markers for salt-alkali tolerance in rapeseed is urgently needed. In this study, we combined

phenotypic data with whole-genome resequencing of 505 accessions under saline-alkali stress to evaluate candidate genes and identify favorable haplotypes. From these analyses, predictive molecular markers were developed, offering a reliable toolset to accelerate the breeding of saline-alkali-tolerant rapeseed varieties. The markers not only enhance our understanding of the mechanisms underlying plant responses to saline-alkali stress but also facilitate the sustainable use of saline-alkali soils.

Materials and methods

1.1 Materials

A total of 505 *Brassica napus* accessions used for whole-genome resequencing were obtained from the National Research Center for Rapeseed Engineering and Technology (Zhang et al. 2021, 2023). The sequencing data are publicly available in the National Genomics Data Center (NGDC) under accession PRJCA008229 (Tang et al. 2021). Comprehensive phenotypic evaluations under salt and saline-alkali stress conditions were performed on the 505 accessions, including measurements of plant height, total dry weight, shoot dry weight, root length, root dry weight, and germination-stage plant height (Table S1). These data were obtained from the *Brassica napus* Multi-Omics Resource Database (Yang et al. 2023).

1.2 SNP calling

Genomic sequence alignment and variant calling were performed using the *Brassica napus* reference genome assembly of cultivar ‘Zhongshuang 11’ (ZS11.v0; https://yanglab.hzau.edu.cn/BnIR/germplasm_info?id=ZS11.v0). Raw sequencing reads were subjected to quality control and adapter trimming with Fastp (v0.20.1) (Chen et al. 2018). High-quality reads were aligned to the reference genome using the BWA-MEM algorithm (v0.7.17) (Li and Durbin 2009). Variant detection was carried out with the Genome Analysis Toolkit (GATK, v4.1.2.0) (McKenna et al. 2010), and a comprehensive variant call format (VCF) file was generated to document all polymorphic loci across the 505 accessions. To ensure data reliability, subsequent variant filtering was performed using PLINK (v1.9) (Purcell et al. 2007) under

stringent thresholds. Specifically, for SNPs, sites with a sample missing rate $\leq 5\%$ and a minor allele frequency (MAF) > 0.05 were retained; for Indels, variants with mapping quality (MQ) ≥ 20 and MAF > 0.01 were included (Zhang et al. 2021)..

1.3 Candidate Gene-Based Association Analysis

Variants located within candidate genes were extracted using PLINK (v1.9) (Purcell et al. 2007) to generate PLINK-formatted datasets for association testing. Association analyses were performed with GEMMA (v0.98) under a linear mixed model (LMM) (Kang et al. 2010; Zhou and Stephens 2012; de Leeuw et al. 2015), while accounting for population structure through a PCA-derived covariate matrix (Price et al. 2006; Alexander et al. 2009). Based on the GEMMA-LMM association results, the proportion of phenotypic variance explained (PVE) by each single nucleotide polymorphism (SNP) was calculated according to the following formula:

$$PVE = \frac{2\beta^2 \times MAF \times (1 - MAF)}{2\beta^2 \times MAF \times (1 - MAF) + se^2 \times 2N \times MAF \times (1 - MAF)}$$

where β represents the allelic effect size estimated by GEMMA, se denotes the standard error of the effect size, N is the sample size, and MAF indicates the minor allele frequency (Shim et al. 2015). Multiple testing correction was applied using the Benjamini-Hochberg procedure, and associations with a false discovery rate (FDR) < 0.05 were considered significant. Significant SNP trait associations were subsequently used to refine the candidate gene set (Storey and Tibshirani 2003)

1.4 Haplotype Analysis of Candidate Genes

Haplotype analysis of candidate genes was conducted using the Multi-Focus Model Variation module implemented on the BnIR online platform (Wang et al. 2025; Yang et al. 2023).

1.5 Development of candidate-gene molecular markers

Gene-based molecular markers were developed at polymorphic sites within candidate genes. Primers were designed using Primer Premier 3 (Premier Biosoft, Palo Alto, CA, USA) to generate SNP markers (amplicon size: 100-300 bp) and

INDEL markers (Untergasser et al. 2012). For KASP (Kompetitive Allele-Specific PCR) assays, DNA sequences encompassing ± 100 bp flanking regions of each target SNP were entered into Primer3 (<http://www.polymarker.info/>) to design a three-primer set per locus, consisting of one common reverse primer and two allele-specific forward primers. The forward primers carried the SNP at the 3' terminus and distinct 5' reporter tails, generating 70-150 bp amplicons (Zhi et al. 2024).

KASP assays were carried out on a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction mixture and PCR cycling conditions followed the standard protocol provided by LGC Genomics. After amplification, plates were read at 25 °C and allele discrimination was performed using the allelic discrimination module in CFX Maestro software (Bio-Rad, Hercules, CA, USA) (Wang et al. 2018).

Results

2.1 Collection and collation of candidate genes

To elucidate the genetic basis of saline-alkali tolerance in rapeseed, we integrated six recent and representative genome-wide association studies (GWAS), which collectively identified 1,723 candidate genes (Table 1). We further identified 99 rapeseed homologs of known salt-tolerance genes from rice, maize, wheat, sorghum, and *Arabidopsis* (Table S2). Phenotypic data covered germination, seedling, and maturity stages, including germination potential (FYS), germination rate (FYL), shoot length (SL), root length (RL), Na⁺ content and high-throughput image-based phenotypes. After mapping these genes to the ZS11 reference genome and removing redundancies, we obtained 1,281 high-confidence genes with well-defined genomic coordinates (Table S3). Variants within these genes were then extracted using PLINK to construct an association dataset, comprising 35,912 variants (Table S4).

Table 1. Sources and origin of candidate genes

Stage	Number	Traits	Candidate gene number	Source
4–5 Leaf-stage	596	High-throughput phenotyping image	234	(Zhang et al. 2023)

Seed Germinati on and Seedling Stages	505	germination potential (FYS), germination rate (FYL), shoot length (SL), and root length (RL), plant height (PH), aboveground dry weight (ADW), and root dry weight (RDW) were measured Determination of Growth Indexes silique length (SL), siliques on main inflorescence (SMI), seeds per silique (SS), thousand-seed weight (TSW), seed yield per plant (SYP), and dry weight per plant (DWP).	405	(Zhang et al. 2021)
The maturity period	146	shoot length (SL), taproot length (RL), and shoot fresh weight (SFW) shoot Na ⁺ content	26	(Zhang et al. 2022)
Seedlings	368	Germination rate (GR), Radicle length (RL), fresh weight (FW), salt tolerance indice was defined as the ratio of the trait under salt stress and the trait under contro	38	(Wan et al. 2017)
germinati on stage	214	The transcriptome analysis and metabolomics analysis	56	(Wan et al. 2018)
Seedlings	2	Root length	53	(Wan et al. 2022)
germinati on	175	germination rates (RGRs)	15	(Chen et al. 2021)
Germinati on	202	germination percentage, shoot length, root length, shoot, dry weight (SDW), Seed vigor index (SVI), stress susceptibility index (SSI), stress tolerance index (STI)	779	(Yang et al. 2024)
The seedling stage	228		117	(Wassan et al. 2021)
Total	2,436		1,723	

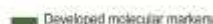
2.2 Candidate gene-based association analysis

Candidate gene association analysis was performed using GEMMA-LMM to estimate the percentage of phenotypic variance explained (PVE) and the corresponding P-value for each variants. Gene-level significance was assessed by applying the Benjamini-Hochberg (BH) procedure to the minimum P-value per gene, adjusted for the number of variants within each gene, yielding a false discovery rate (BH-FDR) for each gene. A total of 483 genes were identified as significant at BH-FDR < 0.05 (Fig. 1; Table S5). Across these associations, we detected 19,455 trait-associated variants, including 6,608 indels (Table S6). Among these, 43 variants individually explained more than 5% of the phenotypic variance. The variant with the largest effect (14.66%) was located in the coding region of *BnaA05G0442900ZS* (Table 2), while the vast majority of variants (~95%) of the variants each contributed

less than 1% (Table S7). These results support a highly polygenic architecture underlying saline-alkali tolerance in rapeseed, suggesting that substantial changes in tolerance are unlikely to arise from variation in only a few genes.

To further evaluate the potential role of these candidate genes under salt-alkali stress, we analyzed RNA-seq data from salt-stressed rapeseed (Wan et al., 2022). In the salt-sensitive cultivar ‘ZS11’, 192 genes were down-regulated and 277 were up-regulated under stress. By contrast, the tolerant cultivar ‘Huayouza 62’ (HZ62) showed 279 down-regulated and 188 up-regulated genes. Using an additional transcriptome dataset, we examined the temporal expression of the 483 candidate genes under salt stress (Chen et al. 2025). The results exhibited that, 156 differentially expressed genes (DEGs) were identified in both 2063A and B409 (Fig. 2A, B), and 129 DEGs were found in HZ62 (Fig. 2C). Notably, 108 genes were differentially expressed across all three materials. (Fig. 2D), reinforcing involvement of these 483 candidate genes in rapeseed salt-stress responses.

Gene Ontology (GO) enrichment analysis (Table S8) showed significant enrichment of biological process (BP) terms related to ion transport, reactive oxygen species (ROS) metabolism, and sodium ion transport. Molecular function (MF) terms were enriched for sodium ion transmembrane transporter activity, potassium ion transmembrane transporter activity, and calcium ion binding. KEGG pathway analysis (Table S9) further identified enrichment in glutathione metabolism, ion channels, arginine and proline metabolism, ascorbate and aldarate metabolism, and fatty acid degradation. These functional annotations strongly support the roles of these candidate genes in mediating rapeseed responses to saline-alkali stress.



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Figure 1. Genome-wide chromosomal distribution of candidate genes

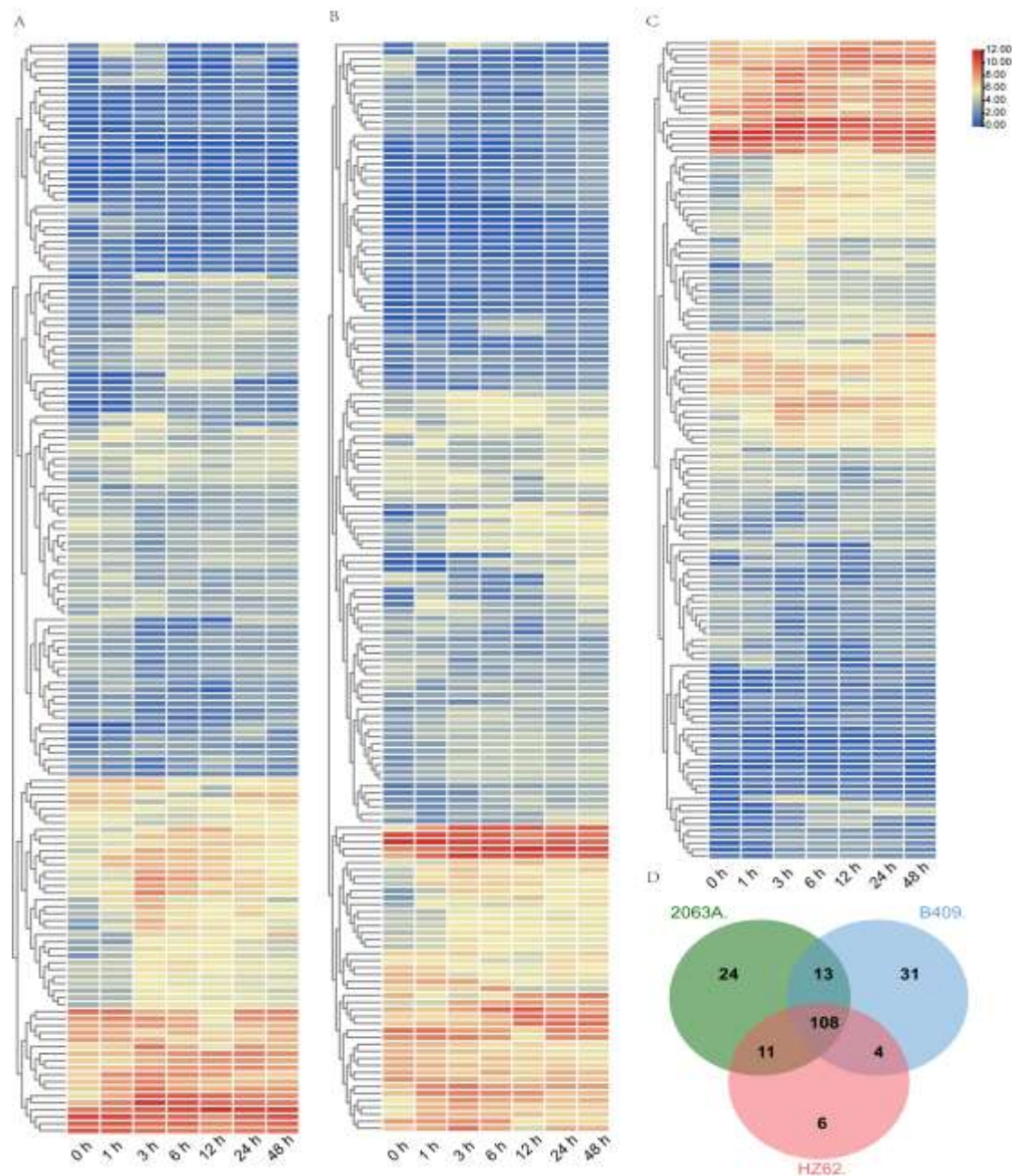


Figure 2. Candidate genes expression profiles under salt stress
 (A): Differential expression of candidate genes in 2063A. (B): Differential expression of candidate genes in B409. (C): Differential expression of candidate genes in HZ62. (D): Venn diagram depicting overlap among differentially expressed candidate genes.

Table 2. PVE >5% of the phenotypic variance

SNP	CHR	A1	A0	Variant class	Gene id	FDR	PVE
A05:41422141	A05	C	A	SNP	BnaA05G0442900ZS	0.0001	14.66%
C03:16112405	C03	A	C	SNP	BnaC03G0260400ZS	0.0021	7.96%
C03:16112466	C03	A	G	SNP	BnaC03G0260400ZS	0.0045	7.16%
C02:18049192	C02	A	G	SNP	BnaC02G0209600ZS	0.0045	6.35%

C01:41699695	C01	T	G	SNP	BnaC01G0362800ZS	0.0045	9.01%
C03:64239622	C03	C	T	SNP	BnaC03G0664700ZS	0.0057	6.45%
C03:16112386	C03	C	T	SNP	BnaC03G0260400ZS	0.0066	6.31%
C03:77758032	C03	A	G	SNP	BnaC03G0800700ZS	0.0066	6.54%
C03:77758028	C03	A	C	SNP	BnaC03G0800700ZS	0.0066	6.53%
C03:77758038	C03	A	G	SNP	BnaC03G0800700ZS	0.0066	6.52%
C03:77758040	C03	C	T	SNP	BnaC03G0800700ZS	0.0066	6.54%
C03:77758010	C03	A	C	SNP	BnaC03G0800700ZS	0.0079	6.27%
C03:77757987	C03	G	A	SNP	BnaC03G0800700ZS	0.0079	6.30%
C01:1453968	C01	A	C	SNP	BnaC01G0026000ZS	0.0089	5.80%
C03:10643674	C03	G	GA	INDEL	BnaC03G0186900ZS	0.0089	6.27%
C03:10643679	C03	C	T	SNP	BnaC03G0186900ZS	0.0089	6.26%
A09:8182840	A09	A	G	SNP	BnaA09G0135100ZS	0.0089	5.44%
C03:77758099	C03	A	G	SNP	BnaC03G0800700ZS	0.0089	5.75%
C04:59545426	C04	A	G	SNP	BnaC04G0468200ZS	0.0089	5.98%
C03:64239684	C03	C	T	SNP	BnaC03G0664700ZS	0.0089	5.23%
C03:64239659	C03	G	A	SNP	BnaC03G0664700ZS	0.0089	5.20%
C03:16112374	C03	T	TTT A	INDEL	BnaC03G0260400ZS	0.0090	5.22%
C03:16112378	C03	C	T	SNP	BnaC03G0260400ZS	0.0090	5.23%
C03:64239653	C03	GG T	G	INDEL	BnaC03G0664700ZS	0.0090	5.17%
C03:77758122	C03	A	C	SNP	BnaC03G0800700ZS	0.0091	5.55%
C03:64239689	C03	A	G	SNP	BnaC03G0664700ZS	0.0100	5.14%
C03:77758115	C03	G	T	SNP	BnaC03G0800700ZS	0.0143	5.20%
C01:5983871	C01	G	A	SNP	BnaC01G0094700ZS	0.0146	5.23%
C03:77758104	C03	C	A	SNP	BnaC03G0800700ZS	0.0150	5.12%
C01:41521211	C01	A	C	SNP	BnaC01G0362000ZS	0.0249	5.42%
C09:38403707	C09	A	AA GCC CAT T	INDEL	BnaC09G0321200ZS	0.0037	8.24%
C09:38403705	C09	C	T	SNP	BnaC09G0321200ZS	0.0042	7.56%
C03:27774544	C03	T	C	SNP	BnaC03G0407000ZS	0.0090	7.47%
A02:4998260	A02	A	ACA C	INDEL	BnaA02G0095400ZS	0.0106	5.51%
C08:52619277	C08	G	T	SNP	BnaC08G0516900ZS	0.0117	7.26%
C09:28817876	C09	T	C	SNP	BnaC09G0294100ZS	0.0169	6.68%
C01:49421306	C01	A	C	SNP	BnaC01G0427700ZS	0.0218	6.66%
A03:3840938	A03	T	C	SNP	BnaA03G0078700ZS	0.0218	5.42%
A03:3840821	A03	T	C	SNP	BnaA03G0078700ZS	0.0218	5.64%
C07:49248770	C07	T	C	SNP	BnaC07G0364700ZS	0.0218	5.69%
A08:15916024	A08	GT	G	INDEL	BnaA08G0098400ZS	0.0081	5.35%
C04:7151376	C04	A	AG	INDEL	BnaC04G0080500ZS	0.0158	6.61%

C04:42634113	C04	T	C	SNP	BnaC04G0315200ZS	0.0055	5.29%
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2.3 Haplotype-based analysis of the candidate genes

Haplotype analysis identified favorable haplotypes in 355 of 483 candidate genes (Table S10), 103 of which were considered rare (frequency < 4). Among the remaining 252 candidate genes, for instance, in *BnaA05G0442900ZS*, hap1 was associated with higher S_PH_R2 values (mean = 0.69, n = 51) compared to hap0 (mean = 0.64, n = 293; $p = 0.0281$) (Fig. 3A, B). Similarly, in *BnaC01G0362800ZS*, hap1 was favorable across three traits—GRL_R1, GRL_T1, and RL_R2 ($p < 0.05$; Table S10). Specifically for RL_R2 (root length under T2/CK), hap1 accessions showed a greater mean value (0.30, n = 96) than hap0 (0.25, n = 200) (Fig. 3C, D).

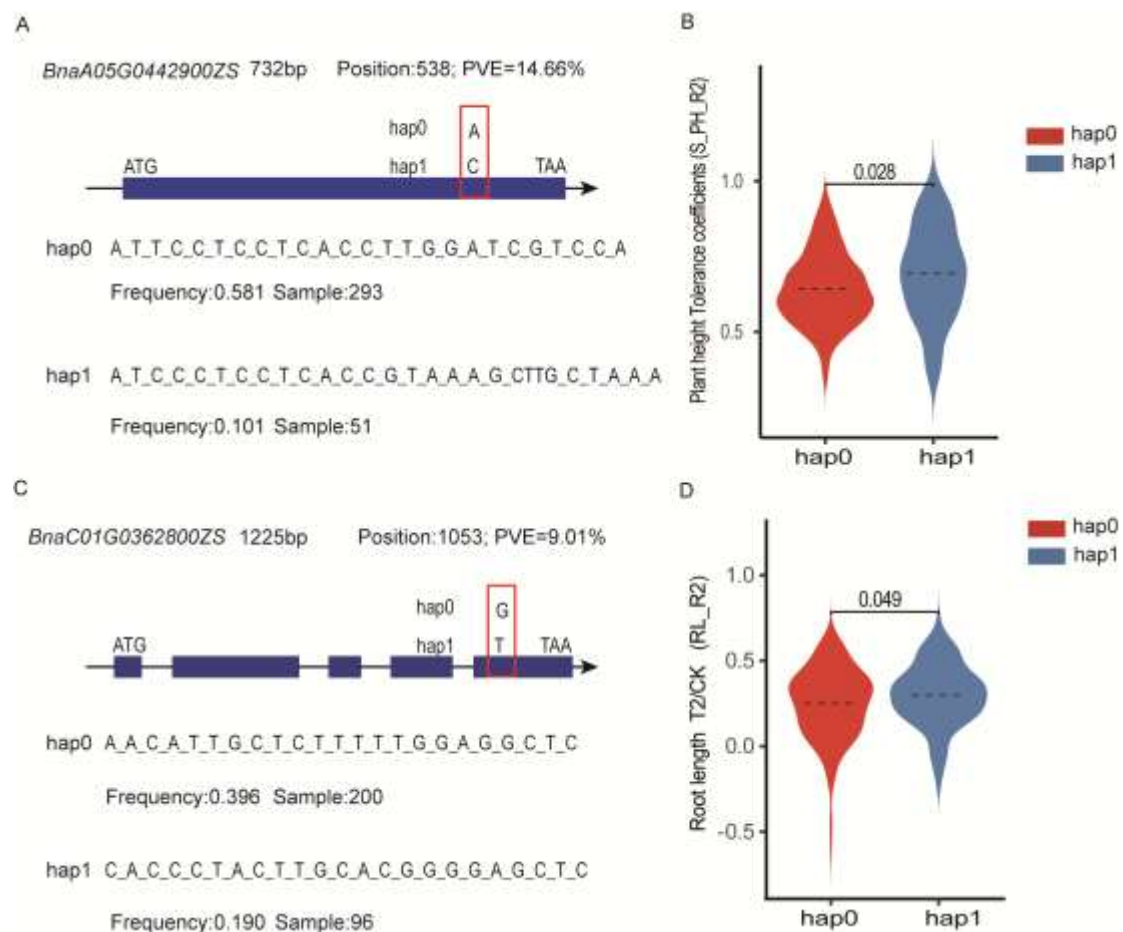


Fig. 3. Effects of *BnaA05G0442900ZS* and *BnaC01G0362800ZS* haplotypes on S_PH_R2 and RL_R2. (A) Major *BnaA05G0442900ZS* haplotypes, hap0 and hap1. (B) Comparison of S_PH_R2 among accessions harboring various haplotypes of *BnaA05G0442900ZS*. (C) Major *BnaC01G0362800ZS* haplotypes, hap0 and hap1. (D) Comparison of RL_R2 among accessions harboring various haplotypes of *BnaC01G0362800ZS*.

2.4 Development and validation of molecular markers for candidate genes

To support marker-assisted selection, we developed 746 KASP assays (Table S11) and 201 InDel markers (Table S12) targeting the 483 candidate genes. Successful assays were established for 275 genes (Figure 1), including 97 genes covered by InDel markers. Chromosome A04 contained the fewest marked genes ($n = 2$), while C03 had the most ($n = 37$). Using 50 accessions each from hap0 and hap1 groups, we validated KASP assays for *BnaA05G0442900ZS* and *BnaC01G0362800ZS*, which accurately distinguished the two haplotype groups (Fig. 4D, E). We further validated InDel markers for *BnaC09G0214000ZS* and *BnaA08G0083300ZS*. In *BnaC09G0214000ZS*, a 50-bp deletion was identified at position 2060 of the reference sequence (Table S14). Genotyping revealed that accessions A21–A32 carried the deletion and yielded no amplification product, whereas A1–A20 produced a band consistent with the reference genotype (Fig. 4A, B). For *BnaA08G0083300ZS*, a 101-bp deletion was detected at position 230. Wild-type samples (e.g., B1, B3, B6–B9) produced two bands (100–300 bp), while mutant samples (e.g., B2, B4, B5, B10–B17) lacked the 100-bp band (Fig. 4C). These results demonstrate that the developed markers enable accurate, genotype-based classification of rapeseed germplasm.

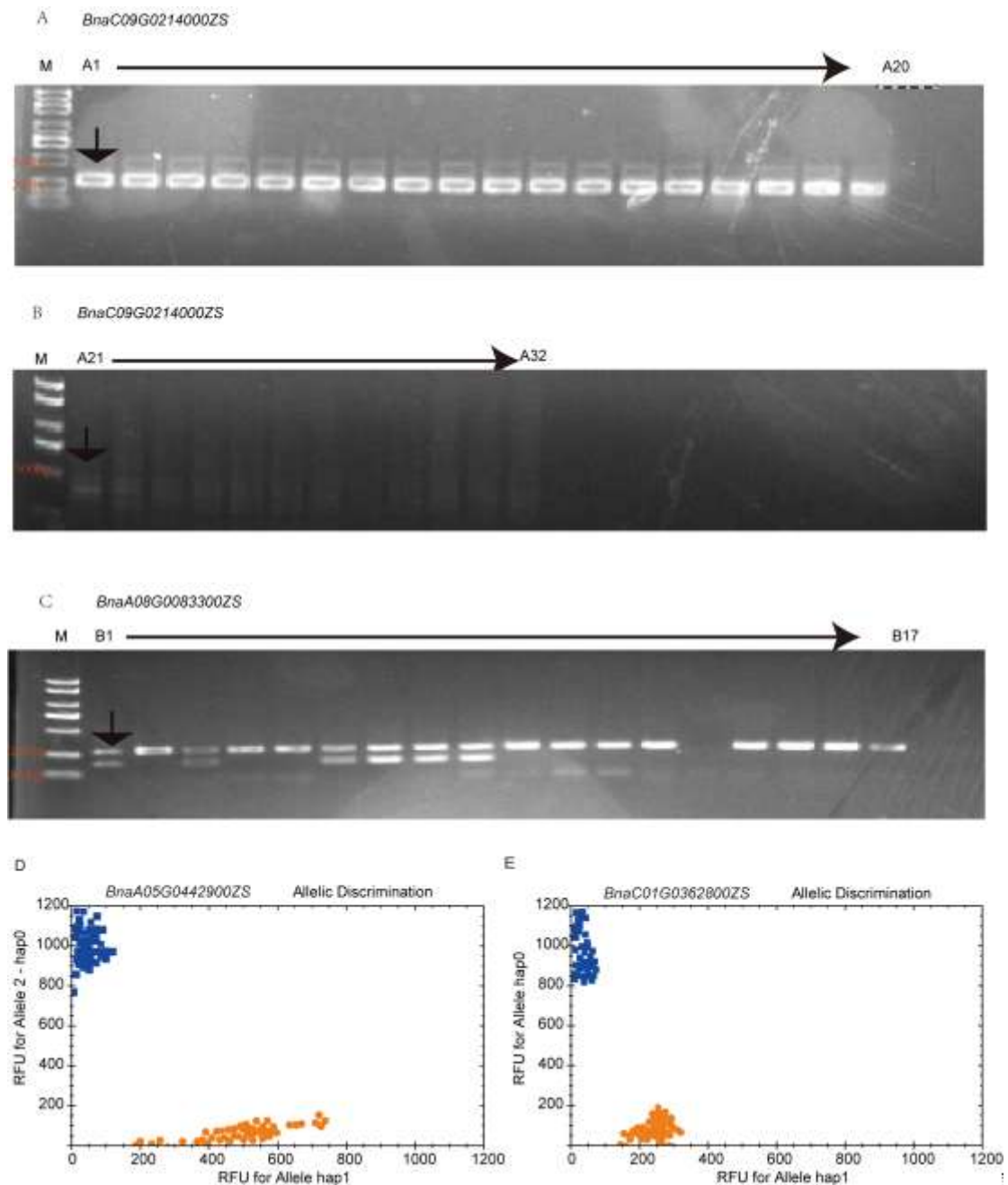


Fig. 4. Indel marker development and validation

(A-B): Validation of the indel marker targeting *BnaC09G0214000ZS*; (M):2000 Mark; (A1-A20): Sample X10, X100, X1000, X1002, X1004, X1012, X1014, X1016, X102, X1020, X1022, X1024, X1026, X1028, X1030, X1032, X1034, X1036, X1038, X104; (A21-A32): Sample X1010, X1018, X1040, X1042, X1046, X106, X114, X128, X132, X146, X156, X168;

(C): Validation of the indel marker targeting *BnaA08G0083300ZS* ; (B1-B17): Sample X12, X120, X122, X124, X128, X130, X132, X134, X136, X198, X20, X232, X240, X264, X284, X318, X342. (D-E): Genotyping map of KASP molecular markers (*BnaA05G0442900ZS* and *BnaC01G0362800ZS*); Blue cluster was a germplasm resource with hap0 allele; orange cluster was a germplasm resource with hap1 allele.

3 Discussion

3.1 Mechanistic divergence of saline-alkali tolerance in rapeseed relative to wheat, rice, and other crops

Under saline-alkali stress, plants are generally classified according to their K^+/Na^+ homeostasis strategies as either Na^+ includers or Na^+ excluders. Rapeseed primarily employs a tissue-based Na^+ sequestration mechanism, storing Na^+ mainly in petioles and stems, whereas wheat relies on a Na^+ exclusion strategy that restricts Na^+ uptake into shoots and confines most Na^+ to the root system (Wang et al. 2025a). Consistent with these physiological differences, salt-tolerant rapeseed cultivars display an enhanced capacity to maintain cytosolic Na^+/K^+ homeostasis and their tolerance typically increases throughout development—in contrast to wheat, where tolerance declines over time (Wang et al. 2022). In rice, HKT-type Na^+ transporters retrieve Na^+ from xylem into parenchyma cells and recirculate it to the roots, reducing shoot Na^+ accumulation under salinity. Meanwhile, non-selective cation channels (NSCCs) contribute to the maintenance of cytosolic Na^+/K^+ balance and participate in salt-induced signaling cascades (Cheeseman 1988; Huang et al. 2008). Collectively, these findings underscore distinct mechanistic adaptations among major crops, with rapeseed relying on tissue-level Na^+ sequestration and cereals primarily employing Na^+ exclusion and retrieval pathways to achieve ionic homeostasis under saline-alkali stress.

GO enrichment analysis revealed that biological processes were primarily associated with potassium ion transport, reactive oxygen species (ROS) metabolism, and sodium ion transport. We further observed that expression of *BnaC02G0368700ZS* (an *HKT1* homolog) was markedly downregulated under salt stress, decreasing 30-fold in the tolerant cultivar HZ62 compared to only 1.5-fold in the sensitive cultivar ZH11 (Table S13). This suggests that rapeseed adapts by sequestering Na^+ and limiting its long-distance translocation—contrasting with rice, wheat, and barley, where upregulated Na^+ transporters often underlie salinity tolerance (Wei et al. 2024). However, it should be noted that *BnaC02G0368700ZS* did

not meet the significance threshold ($\text{FDR} > 0.05$) in our association analysis (Table S15). Among the 483 candidate genes identified, 73.3% contained four or more homeologs, and four-copy loci were the most prevalent (20.3%), further indicating that rapeseed employs more complex genetic mechanisms to cope with saline-alkali stress compared to other crops.

Recent studies have begun to elucidate the distinctive mechanisms underlying salt tolerance in oilseed rape. For instance, *BnaWIP2* promotes seed germination under salinity by downregulating abscisic acid (ABA) biosynthesis and signaling (He et al. 2025), while *BnBBX22.A07* enhances tolerance through activation of ROS-scavenging pathways and reduction of salt-induced ROS accumulation. Consistent with these insights, integrated phenotyping and multi-omics analyses of 30 winter accessions identified phenylalanine biosynthesis and arginine/proline and cysteine/methionine metabolism as central pathways supporting salt tolerance (Ma et al. 2025). KEGG enrichment analysis further indicated that the 483 candidate genes were involved in glutathione metabolism, ion channel activity, arginine and proline metabolism, ascorbate metabolism, and fatty acid degradation — collectively supporting their role in saline-alkali stress responses. Additional KEGG terms included amino sugar and nucleotide sugar metabolism, porphyrin metabolism, ether lipid metabolism, proteasome function, and endoplasmic reticulum protein processing, suggesting that tolerance is also achieved through osmotic adjustment and the stabilization of protein and membrane integrity.

Under salt stress, rapeseed displays reduced trichome and stomatal density, plasmolysis, and chloroplast degradation, along with increased levels of anthocyanins, osmolytes, ABA, jasmonic acid (JA), pectin, cellulose, ROS, and enhanced antioxidant capacity. By contrast, photosynthetic pigments, indole-3-acetic acid (IAA), cytokinins, gibberellins, and lignin decrease (Feng et al. 2020). Up-regulation of *BnaNHXs* (vacuolar Na^+/H^+ antiporters) further promotes vacuolar Na^+ sequestration (Cui et al. 2020). These findings indicate that rapeseed relies primarily on vacuolar ion sequestration, membrane-lipid remodeling, and integrated hormone signaling—

rather than solely on Na⁺ efflux or vascular exclusion—thereby marking a clear mechanistic divergence from wheat and rice and opening new research avenues for improving saline-alkali tolerance in crops.

3.2 Pyramiding Multiple Salt-Alkali Tolerance Loci to Improve Rapeseed Resilience

Salt-alkali tolerance in rapeseed is a typical polygenic trait. Most loci exert only small individual effects and are strongly shaped by genotype-environment interactions. Given this complex architecture, the heterotic expression of stress-responsive genes becomes an important contributor to hybrid performance. Previous work has shown that hybrids display a range of non-additive genetic effects, including full dominance, partial dominance and overdominance, which collectively enhance stress adaptation (Chen et al. 2025). In our study, nearly 95% of the detected variants explained less than 1% of the phenotypic variation, which confirms that saline-alkali tolerance cannot be substantially improved by modifying only a few genes.

Hybrid performance therefore depends on the accumulation of favorable alleles that originate from both parents. This observation supports a breeding strategy that focuses on pyramiding multiple loci rather than seeking breakthroughs based on single major-effect genes. The combined assembly of beneficial alleles related to ion homeostasis, osmotic adjustment and antioxidant regulation can greatly strengthen the resilience of hybrid cultivars under saline – alkaline stress.

The 947 KASP and InDel markers developed in this study provide a solid foundation for implementing molecular design breeding for saline-alkali tolerance in rapeseed. These markers support the development of superior hybrids through multilocus allele pyramiding. Specifically, they guide parental selection and cross-design decisions to optimize allele combinations. The markers also facilitate early-generation identification of favorable haplotypes and assist in identification of valuable germplasm, thereby significantly enhancing breeding efficiency. Integrating this marker system into large-scale genomic design and prediction workflows will help move breeding for saline – alkali tolerance from empirical selection toward a more precise, genotype-informed approach, thereby accelerating the development of

improved tolerant cultivars.

4. Conclusions

Integrated candidate gene-based association analysis, transcriptome profiling, and GO/KEGG enrichment revealed that rapeseed exhibits uniquely complex stress-response mechanisms under saline-alkali conditions, distinct from those in other crops. Among the 1,698 saline-alkali stress-related genes analyzed, 483 were significantly associated, with 355 carried favorable haplotypes. Molecular markers were developed for 275 genes and experimentally validated, enabling reliable germplasm classification and preliminary evaluation of saline-alkali tolerance, and providing a key genomic resource that will drive future precision breeding and multi-gene pyramiding strategies in rapeseed.

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Contributions

X. H., J. S., and Y. G. designed the research and wrote the article. F. L., O. L., X. G., C. H., C. H., R. J., J. C., M. Z., D. J., Q. L., H. M., J. W., L. Z., H. W., and T. F. performed the research. X. H. analyzed the data. All authors have read and approved the final version of the manuscript.

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Ethics declarations

This article does not contain any studies with human or animal subjects

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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