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RESEARCH ARTICLE

Genome-wide association analysis of aluminum tolerance related traits in rapeseed (*Brassica napus* L.) during germination

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Abstract With the increasing acidification of soil, aluminum (Al) toxicity has become one of the most important stress factors affecting seed germination quality and crop yield. To investigate the Al tolerance on seed germination, genome-wide association analysis (GWAS) of 19,949 SNPs with genome-wide coverage was used to identify the candidate genes, which were potentially related to germinate traits of rapeseed *(Brassica napus L.)* under Al stress. In the experiment, 169 rapeseed cultivars (lines) were treated with $AlCl_3$ solution of 90 ppm, and distilled water was added to the control. At the 7th day, the phenotype data, including root length and dry weight, were measured and calculated. Using the TASSEL software, Al tolerance related traits were explored in rapeseed under germination with a 60 K Brassica Illumina® Infinium SNP array. Then, the structure of the population was analyzed with the STRUCTURE software, and the genetic relationship and LD attenuation were analyzed with the software TASSEL, respectively. The GWAS of relative root length (RRL) and relative dry weight (RDW) with SNP markers were carried out under the optimal model. Meanwhile, the candidate genes were predicted based on the LD interval sequence of the associated SNP locus. Subsequently, the homologous genes of rapeseed related to Al tolerance in the target genome region were screened in *Arabidopsis thaliana* (L.) Heynh. The results showed that 13 SNPs were significantly associated with these two traits. Among them, 8 SNPs were significantly associated with RRL and located on chromosomes A03, A07, A09, A10, C05, C06, and C09, respectively. Five SNPs were significantly associated with RDW and located on chromosomes A03, A04, A10, C05, and C07, respectively. Afterward, fifty-nine candidate genes related to Al tolerance were identified in the LD region of these SNP loci. Four of these genes were involved in the growth regulation about organic acid, ten were involved in growth-regulating substance, eleven were related to oxidative stress, and nineteen were involved in carbon and nitrogen metabolism. The results of this study provided a theoretical basis for Al tolerance in rapeseed and laid out a foundation for further functional verification of genes and cultivation of new Al tolerant rapeseed varieties.

Keywords GWAS · *Brassica napus* L. · Germination · Aluminum stress

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Introduction

Aluminum (Al) is the most prevalent metal and the third most abundant element in the earth's crust (Exley 2009). Al in solid or bound form does not cause toxicity to plants, environment, and human beings, but ionic (Al^{3+}) or free Al [$Al(OH)^{2+}$] can cause great toxicity (Kochian 1995). With the application of ammonium fertilizer and the increasing acid rain in recent years, the expanded area of acidic soil directly caused an increased dissociation of Al^{3+} ions (Meng et al. 2017). Studies are indicating that increasing dissociated Al^{3+} ions can damage the cell activity of root meristem, inhibit cell mitosis, root growth (Marciano et al. 2010; Doncheva et al. 2005), affect the uptake of nutrition (Li et al. 2015), and ultimately limit crop growth and yield (Furukawa et al. 2007). To alleviate the harm of Al toxicity, many efforts have been made, especially in the genetic mechanism and gene improvement. Variations in Al-tolerant genes in wheat (Hamel et al. 1998), barley (Cai et al. 2013), rice (Nguyen et al. 2001), oat (Castilhos et al. 2011), sorghum (Gourley et al. 1990), and maize (Mattiello et al. 2012) have been guessfully reported. Then high-yielding varieties with stronger tolerance to Al toxicity have been developed in breeding programs. Rapeseed (*Brassica napus* L.) is considered a plant with weak tolerance to Al (Qian et al. 2014), and it's mainly planted in the Middle and Lower parts of Yangtze River, the regions with more acidic soil in China. Therefore, Al toxicity has become an important stress factor in its production (Ryan and Delhaize 2010). Seed germination is the initial stage of the plant life cycle, which directly affects the growth and development as well as the production benefits of rapeseed. Exploring the genetic mechanism of Al tolerance in rapeseed during germination is of great significance to the cultivation of high-yielding varieties of Al tolerant rapeseed.

Genome-wide association study (GWAS) was first applied to human disease research, and then gradually applied to plant research. The continuous improvement of SNP marker mining technology covering the whole genome and GWAS based on Linkage Disequilibrium (LD) provided a new way of controlling complex traits in diverse natural populations (Gupta et al. 2014). Atwell et al. (2010) demonstrated the feasibility of GWAS application in *Arabidopsis thaliana* (L.) Heynh. for the first time and pointed out that this method can also be applied to other plants (Atwell et al. 2010). At present, GWAS has been widely used in studying crop stress resistance, such as salt tolerance (Shi et al. 2017), cold tolerance (Huang et al. 2013a), and drought-induced stress (Li et al. 2018). GWAS studies on Al tolerance in crops have also been reported. Tao et al. (2018) identified a total of 21 candidate genes for 7 important QTL regions associated with Al toxicity tolerance in rice. Cai et al. (2013) carried out GWAS on root length Al tolerance of Tibetan wild barley and cultivated some varieties based on SNP markers. With the publication of the whole genome sequence of rapeseed and the development of 60 K Illumina Infinium SNP chip, the application of GWAS in rapeseed has also made significant advances. Some loci associated with important agronomic and quality traits of rapeseed were identified (He et al. 2017; Liu et al. 2016; Huang et al. 2013b; Xue et al. 2018; Wei et al. 2016). Also, GWAS was already carried out on germination-related indexes such as germination vigor and rate in rapeseed (Hatzig et al. 2015; Tan et al. 2017). However, few GWAS studies were conducted on Al tolerance to rapeseed during germination. In this study, 169 rapeseed cultivars (lines) were treated with Al solution at seed germination stage. GWAS analysis of relative root length and relative dry weight was done to reveal the genetic and molecular mechanisms of Al tolerance in rapeseed, which will provide references for the cultivation of high-yield varieties with Al tolerance.

Materials and methods

Materials and reagents

One hundred and sixty-nine rapeseed germplasms, with different genetic backgrounds, and broad geographical origins, were collected as materials from universities and research institutes in China (Table 1). All varieties (lines) were provided by Chongqing Engineering and Technology Research Center for Rapeseed, China (106.40° E, 29.80° N).

The treatment agent was $AlCl_3 \cdot 6H_2O$ with 97% analytical purity, which was produced by Chengdu Chron Chemicals Co., Ltd., Sichuan province, China.

Table 1 Information of 169 rapeseed cultivars (lines)

Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources
Ganyou5	Chongqing, China	WH-41	Hubei Province, China	9F087	Hubei Province, China	Suyou1	Jiangsu Province, China
Yan81-2	Chongqing, China	WH-45	Hubei Province, China	97,097	Hubei Province, China	Zheshuang8	Jiangsu Province, China
SWU40	Chongqing, China	WH-49	Hubei Province, China	7037	Hubei Province, China	Zheyou758	Jiangsu Province, China
SWU46	Chongqing, China	11-7-117	Hubei Province, China	RQ011	Hubei Province, China	Huayou19	Jiangsu Province, China
SWU47	Chongqing, China	WH-60	Hubei Province, China	97,177	Hubei Province, China	Zheyou21	Jiangsu Province, China
SWU48	Chongqing, China	Jiayu17peng	Hubei Province, China	96,021	Hubei Province, China	Wanyou29	Jiangsu Province, China
SWU56	Chongqing, China	Jiayu25peng	Hubei Province, China	96,063	Hubei Province, China	Y2	Jiangsu Province, China
SWU59	Chongqing, China	Jiayu31peng	Hubei Province, China	1111	Hubei Province, China	Sahoyeqing	Jiangsu Province, China
SWU65	Chongqing, China	Jia972	Hubei Province, China	1570	Hubei Province, China	Hujizao	Jiangsu Province, China
SWU92	Chongqing, China	Jia915	Hubei Province, China	9 bao 22	Hubei Province, China	Dahuaqiu	Jiangsu Province, China
SWU101	Chongqing, China	Jia922	Hubei Province, China	1188	Hubei Province, China	Rongxuan	Jiangsu Province, China
IMC103	Chongqing, China	Jia917	Hubei Province, China	2354	Hubei Province, China	Ningyou6	Jiangsu Province, China
Oscar	Chongqing, China	Jia931	Hubei Province, China	2359	Hubei Province, China	Guangde8104	Jiangsu Province, China
campina	Chongqing, China	Jia963peng	Hubei Province, China	93,205	Hubei Province, China	Su84-6	Jiangsu Province, China
Youyan2	Chongqing, China	09-P32	Hubei Province, China	93,210	Hubei Province, China	Wanyouzao	Jiangsu Province, China
SWU49	Chongqing, China	10-P10	Hubei Province, China	Nca	Hubei Province, China	Jie65-1	Jiangsu Province, China
SWU64	Chongqing, China	11-P30	Hubei Province, China	Zhongshuang4	Hubei Province, China	wx1025	Hunan Province, China

Table 1 continued

Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources
SWU69	Chongqing, China	WH-19	Hubei Province, China	Zhongshuang11	Hubei Province, China	wx10213	Hunan Province, China
SWU71	Chongqing, China	WH-20	Hubei Province, China	2011-6308	Hubei Province, China	wx10315	Hunan Province, China
SWU75	Chongqing, China	WH-28	Hubei Province, China	2012-3448	Hubei Province, China	10-1358	Hunan Province, China
SWU77	Chongqing, China	WH-55	Hubei Province, China	2012-3546	Hubei Province, China	Xiangyou13	Hunan Province, China
SWU80	Chongqing, China	WH-95	Hubei Province, China	2012-5086	Hubei Province, China	782	Hunan Province, China
SWU81	Chongqing, China	NY7	Hubei Province, China	2012-8380	Hubei Province, China	WX10329	Hunan Province, China
SWU84	Chongqing, China	Huayou6	Hubei Province, China	2012-8998	Hubei Province, China	Santana	Hunan Province, China
SWU88	Chongqing, China	Huayou10	Hubei Province, China	2012-9478	Hubei Province, China	509	Hunan Province, China
SWU94	Chongqing, China	Suigenshu	Hubei Province, China	Xiwang106	Hubei Province, China	1321	Hunan Province, China
SWU95	Chongqing, China	Huayou4	Hubei Province, China	Yangguang198	Hubei Province, China	A117	Shanxi Province, China
SWU96	Chongqing, China	7022	Hubei Province, China	Zhongshuang10	Hubei Province, China	B250	Shanxi Province, China
SWU99	Chongqing, China	7094	Hubei Province, China	Zhongshuang12	Hubei Province, China	B265	Shanxi Province, China
SWU100	Chongqing, China	Chuanyou20	Sichuan Province, China	Zhongyou589	Hubei Province, China	A109	Shanxi Province, China
SWU110	Chongqing, China	Chuanyou18	Sichuan Province, China	1L187	Hubei Province, China	GY284	Shanxi Province, China
SWU111	Chongqing, China	CY12Q95406	Sichuan Province, China	Zhongshuang6	Hubei Province, China	A97	Shanxi Province, China
SWU112	Chongqing, China	CY12Q8-7	Sichuan Province, China	1L191	Hubei Province, China	A148	Shanxi Province, China
Huayou13	Hubei Province, China	CY12QSZ06	Sichuan Province, China	Fuyou4	Hubei Province, China	03IIB	Gansu Province, China

Table 1 continued

Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources	Varieties (lines)	Sources
Rucabo	Hubei Province, China	CY12Q95108	Sichuan Province, China	Zhongshuang11	Hubei Province, China	964	Gansu Province, China
Ningyou1	Hubei Province, China	CY12Q21535-N3	Sichuan Province, China	YangJ6711	Hubei Province, China	DDI	Gansu Province, China
11–9-704	Hubei Province, China	CY18PXW-62	Sichuan Province, China	Zheyou17	Hubei Province, China	06T9F	Gansu Province, China
11–9-705	Hubei Province, China	Huyou17	Jiangsu Province, China	De68-12	Hubei Province, China	03II4B	Gansu Province, China
11–9-706	Hubei Province, China	Huyou15	Jiangsu Province, China	Monty	Hubei Province, China	03LF1	Gansu Province, China
11–9-707	Hubei Province, China	Ningyou14	Jiangsu Province, China	Shilifeng	Hubei Province, China	9852	Gansu Province, China
WH-33	Hubei Province, China	Yangyou6	Jiangsu Province, China	Ningyou18	Hubei Province, China	06H7	Gansu Province, China
WH-37	Hubei Province, China	Yangyou5	Jiangsu Province, China	Qing662A	Hubei Province, China	Zhizun	Qinghai Province, China
WH-38	Hubei Province, China						

Design and phenotyping

In each variety (line), the uniform-size and full seeds were selected for the germination experiment. Then, 20 grains that were chosen from every variety (lines) and then washed with distilled water three times were evenly placed in Petri dishes on filter paper, respectively. 3 mL distilled water was added in every Petri dish for control and 3 mL Al solution (pH = 4.0) with a concentration of 90 ppm was added for stress (Gao et al. 2019). Each treatment was repeated three times, and the solutions were replaced every 2 days. All the materials were incubated at 25 °C under a photoperiod of 16 h day/8 h night and 85% relative humidity.

On the 7th day of seed germination, 10 seedlings with similar growth were randomly selected from each dish to determine their root length and dry weight. Then the relative root length (RRL) and relative dry weight (RDW) of each variety (lines) were calculated as the ratio of treatment to control for GWAS analysis (Gao et al. 2019).

Data processing was carried out with Microsoft Excel 2016 software, while variance analysis and principal component analysis was performed with DPS 2005 software (Tang and Zhang 2013).

Genotype determination and analysis

60 k Illumina Infinium SNP chip was used to carry out the genotype analysis of 169 materials of rapeseed (Unterseer et al. 2014) using the Genome Studio version 2011.1 software (Illumina company, https://www.illumina.com.cn). SNP markers with more than 20% missing calls, and minor allele frequency lower than 0.05 or heterozygosity rate more than 20% across the panel were excluded. After preprocessing, 19,949 high-quality SNP markers with a unique physical location in the rapeseed genome were obtained for linkage disequilibrium (LD) and population structure

analysis. The threshold in this study was set at 4.30 [− log (1/19,949)].

Genome-wide association analysis

According to 19,949 unlinked SNPs on 19 chromosomes of rapeseed, the population structure of the 169 rapeseed varieties (lines) was analyzed with STRUCTURE 2.3.4 software (Pritchard et al. 2000). Afterward, the results were added into the website of STRUCTURE HARVESTER (https://taylor0.biology.ucla.edu/structureHarvester/), and the appropriate ΔK value was chosen to determine the number of subgroups and obtaining Q matrix (Earl and Holdt 2012). The parameter settings for estimation of membership coefficients for varieties in each subpopulation were a burn-in of 10,000 generations followed by 100,000 iterations for each of the clusters (K) from 1 to 10, with each K running five times. The most likely K-value was determined by the log probability of the data [ln P(K)] and the value of ΔK, based on the rate of change of ln P(K) between runs using successive K-values as described by Earl et al. (2012). The maximum membership probability among subgroups was applied to divide the accessions into different subgroups. The relationship among materials was analyzed in the kinship module of TASSEL 5.1.0 to obtain K matrix (Kinship) and PCA matrix (Principal Component Analysis) (Bradbury et al. 2007). Finally, combined with 19,949 high-quality SNP markers selected, GWAS built six models of the general linear model (GLM) and mixed linear model (MLM) with Q, PCA and K matrices as covariates.

The LD decline plot of each chromosome was plotted against the physical distance with TASSEL 5.1.0 using the full matrix and sliding window options for sub-genomes A and C (Bradbury et al. 2007). With the determinant coefficient R^2, representing the attenuation threshold, 0.2, the LD decline distances of the chromosomes significantly associated with SNPs were calculated.

Annotation of candidate genes

According to the position of LD interval in rapeseed genome, the number of genes and the sequence of gene-coding proteins in LD interval were analyzed based on the genome annotation information of rapeseed “Darmor-Bzh,” published in France (https://www.genoscope.cns.fr/brassicanapus) (Li et al. 2016). The gene expression patterns, possible co-expression genes, and interacting proteins of *Arabidopsis thaliana* homologous genes were analyzed in the website of Arabidopsis Information Resource (https://www.arabidopsis.org/). With the E-value threshold at 1×10^{-10}, functions of candidate gene were annotated according to *Arabidopsis thaliana* genes with the highest homology.

Results

Phenotypic data

The RRL and RDW of the 196 rapeseed varieties (lines) exhibited extensive phenotypic variation under Al stress (Table 2). The RRL ranged from 0.271 to 1.874 with the mean as 0.705, while RDW ranged from 0.500 to 1.821 with the mean as 0.964. The variation coefficient of RRL and RDW was 49.656% and 15.366%, respectively. Frequency analysis of RRL and RDW showed that these two traits had a continuous distribution (Fig. 1), which was in accordance with quantitative traits and was suitable for GWAS analysis.

Population structure and genetic relationship

The highest ΔK was observed when K = 2 (Fig. 2A). Accordingly, the population could be divided into two subpopulations: P1 (46 accessions) and P2 (123 accessions) (Fig. 2B). The relative kinship analysis revealed that the population of rapeseed had a null or weak relationship, with 55.06% not related (Fig. 2C). The results indicated that GWAS analysis could be performed due to the distant genetic relationship among the tested population materials.

Linkage disequilibrium (LD)

The LD of sub-genome A and C of rapeseed was estimated according to the R^2 among SNP markers. The attenuation distances of sub-genomes A and C were different, but their R^2 decreased with the extension of genetic distance (Fig. 3). In sub-genome A, the attenuation distance of chromosome A08 was the longest, about 1250 Kb, A09 and A10 chromosomes were about 500 Kb and 330 Kb, respectively.

Table 2 Phenotypic statistics of RRL and RDW under Al tolerance

Traits	Maximum	Minimum	Mean ± standard deviation (Mean ± SD)	Coefficient of variation CV (%)
RRL	1.874	0.271	0.705 ± 0.257	49.656
RDW	1.821	0.500	0.964 ± 0.148	15.366

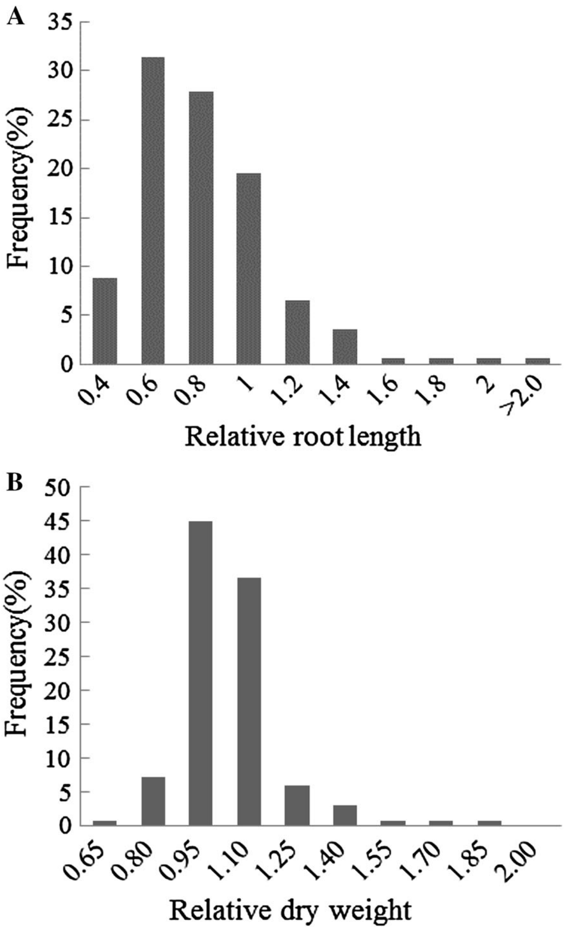

Fig. 1 Phenotype frequency distribution. **A** Frequency distribution of RRL; **B** frequency distribution of RDW

The others followed, mostly around 150–200 Kb with little change. In sub-genome C, the attenuation distance of chromosome C02 was about 1490 Kb, obviously longer than other chromosomes, indicating that there were more genes affecting traits selected by artificial evolution in chromosome C02. Simultaneously, the attenuation speed of chromosome C05 was the slowest, with an attenuation distance around 260 Kb. The average attenuation distance of sub-genome A was 329 Kb with $R^2 = 0.2$, and sub-genome C was 730 Kb (Table 3). The reason that the decline rate of sub-genome A was slower than the one of sub-genome C could be related to the large-scale recombination of sub-genome A in Chinese semi-winter rapeseed and the breakdown of linkage imbalance (Lu et al. 2016).

Genome-wide association analysis

General linear models (GLM) including GLM, Q, and PCA models as well as mixed linear models (MLM), including K, K + Q, and K + PCA models were made with GWAS. When comparing the Q-Q plots (Fig. 4) of distribution under these six models, the K + PCA models of RRL and RDW in MLM were found closer to the predicted line, so the K + PCA model was selected to find the related loci.

Eight SNP markers related to RRL were identified on chromosomes A03, A07, A09, A10, C05, C06, and C09, with phenotypic variation explained (PVE) ranging from 14.566% to 26.102% (Table 4, Fig. 5A). Five SNP markers related to RDW were identified on chromosomes A03, A04, A10, C05, and C07, which explained 14.679–17.83% of the phenotypic variation (Table 4, Fig. 5B).

Potential candidate genes

Through GWAS analysis, the genes significantly associated with RRL and RDW were identified in the LD interval of SNP loci. Then according to the gene function predicted with the Arabidopsis Information Resource (Xu et al. 2016), fifty-nine candidate genes related to Al tolerance were ultimately screened out (Table 5).

Through GWAS analysis of RRL, 31 candidate genes related to Al tolerance were the most reliable, distributed on chromosomes A03 (3), A07 (1), A09 (3), A10 (4), C05 (1), C06 (7), and C09 (12), respectively. Among them, two MATE family

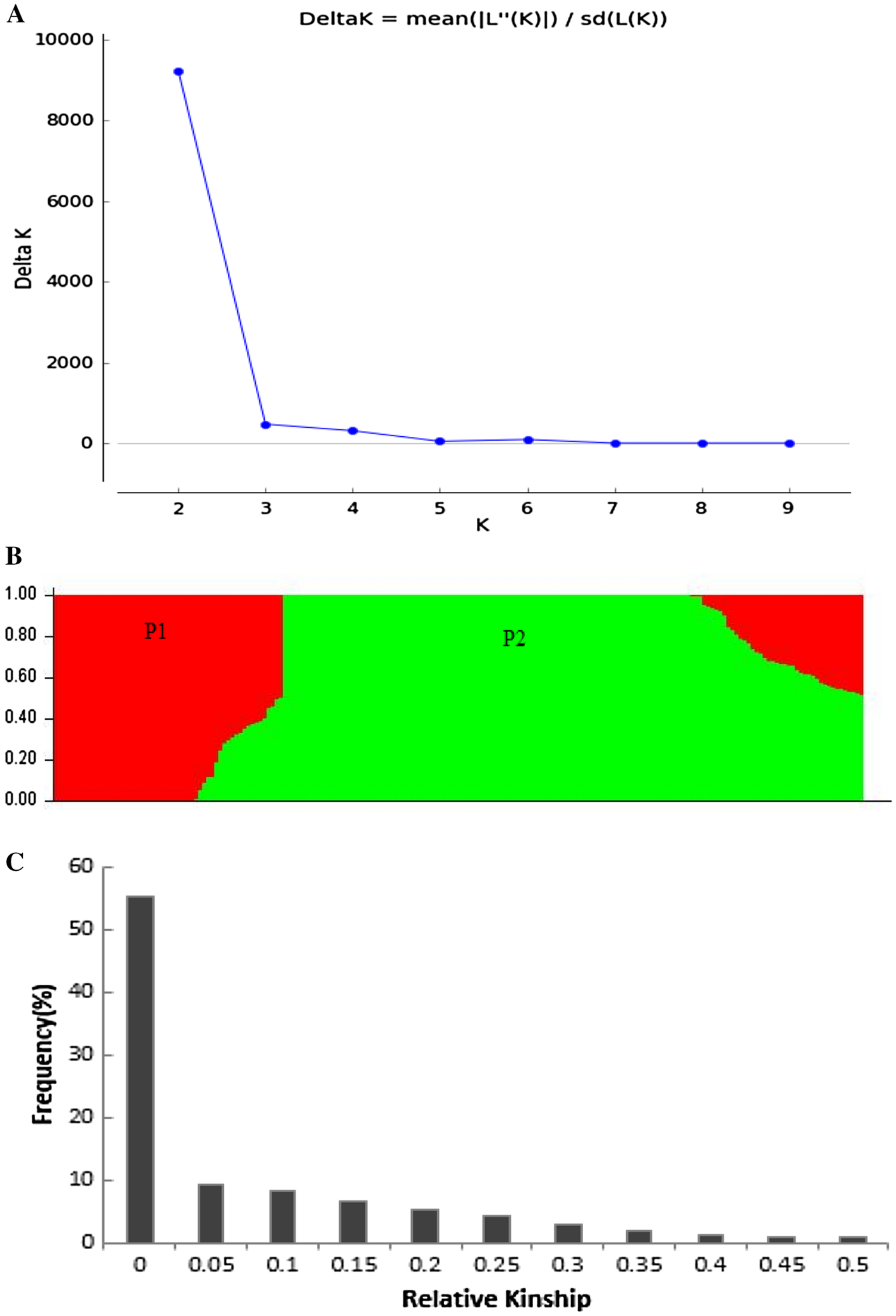

Fig. 2 Analysis of population structure and relative kinship in 169 *Brassica.napus*. **A** Estimation of Δk value in population; **B** group structure diagram; **C** distribution of relative kinship values

proteins located on chromosomes A03 and C09 were found, namely *BnaA03g51020D* and *BnaC09g40230D*. MATE transporter *ABS4* (*BnaA09g14730D*) located in chromosome A09 was found, which could alleviate Al toxicity by regulating the citric acid synthesis pathway. Besides, *BnaA10g20060D* (*JAZ10*) on chromosome A10 and *BnaC09g40420D* (*RGL3*) on chromosome C09 were found to participate in jasmonic acid (JA) signal regulation. Also, *BnaC09g40420D* (*RGL3*) took part in salicylic acid (SA) signal transduction together with *BnaC09g41020D* (*OPR1*) on chromosome C09. A

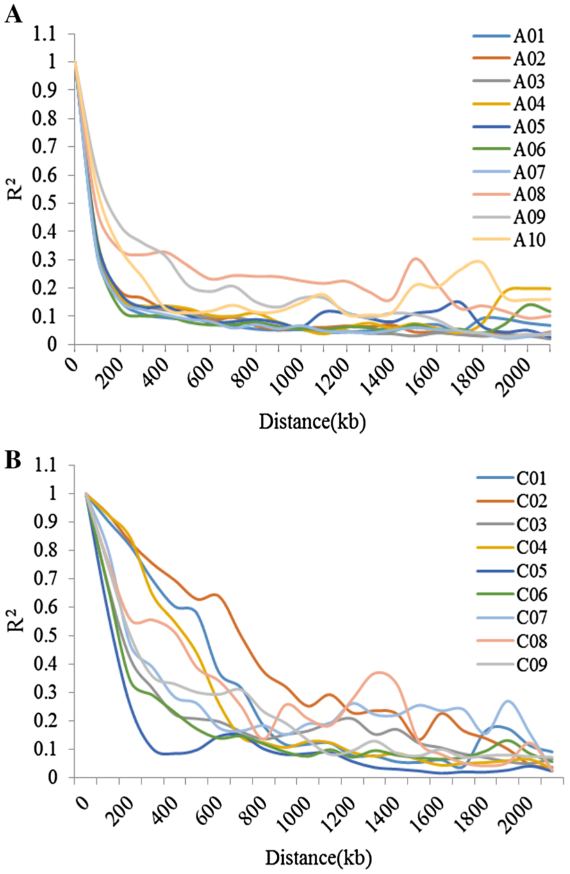

Fig. 3 The linkage disequilibrium declines in different chromosomes for sub-genome A and C. **A** LD decay of sub-genome A; **B** LD decay of sub-genome C

gene called *BnaA09g13940D* (*GA20OX4*) located on chromosome A09 was found to encode gibberellin oxidase. A gene called *AT3G53280* (*CYP71B5*) was screened out, which belonged to the cytochrome P450 family. Some genes that participated in carbohydrate metabolism were screened out, including *BnaA03g51300D* (O-Glycosyl hydrolases family 17 protein) on chromosome A03, *BnaA10g19920D* (*TRA2*) on chromosome A10, as well as *BnaC09g39650D* (*UTP*), *BnaC09g40390D* (*MEX1*), and *BnaC09g40960D* (*CSLD2*) on chromosome C09. They mostly involved glucose, galactose and maltose metabolism, cellulose polysaccharide biosynthesis, and starch catabolism. When it came to nitrogen compound metabolism, genes were found in *BnaC06g14440D* (*NODGS*) on chromosome C06 and *BnaC09g39680D* (*GDH1*) on chromosome C09.

Table 3 LD attenuation distance of chromosomes for sub-genome A and C

Chromosome	Chr-A LD attenuation distance (Kb)	Chromosome	Chr-C LD attenuation distance (Kb)
A01	200	C01	720
A02	200	C02	1490
A03	150	C03	600
A04	160	C04	700
A05	190	C05	260
A06	150	C06	480
A07	160	C07	600
A08	1250	C08	800
A09	500	C09	920
A10	330		
Chr-A mean	329	Chr-C mean	730

Through GWAS analysis of RDW, 28 candidate genes related to Al tolerance were screened out, distributed on chromosomes A03 (5), A04 (4), A10 (6), C05 (4), and C07 (9), respectively. Among them, *BnaC07g27050D* on chromosome C07 was found to belong to the MATE family gene. Two genes of BnaA03g15910D (*JAZ7*) on chromosome A03 and *BnaA04g10280D* (*MKK3*) on chromosome A04 involved in JA signal regulation were found. *BnaA03g15880D* (*ATGA2OX3*) and *BnaA10g23640D* (*GA20OX3*) on chromosome A10 encoded gibberellin oxidase and participated in oxidative stress response. *BnaC07g26120D* (*ERD5*) on chromosome C07 took part in oxidation and was also related to proline decomposition. BnaA03g16180D on chromosome A03 belonged to copper amine oxidase family protein and participated in nitrogen metabolism. Besides, 11 genes located on chromosomes A03 (1), A04 (3), A10 (1), C05 (1), and C07 (5) were found and involved in carbon metabolism, all of which involved starch and sucrose biosynthetic metabolism, and glycogen decomposition.

Discussion

SNP loci

The development of SNP chip provides a new channel for exploring genes and loci related to crop stress tolerance. It has been widely used in GWAS analysis,

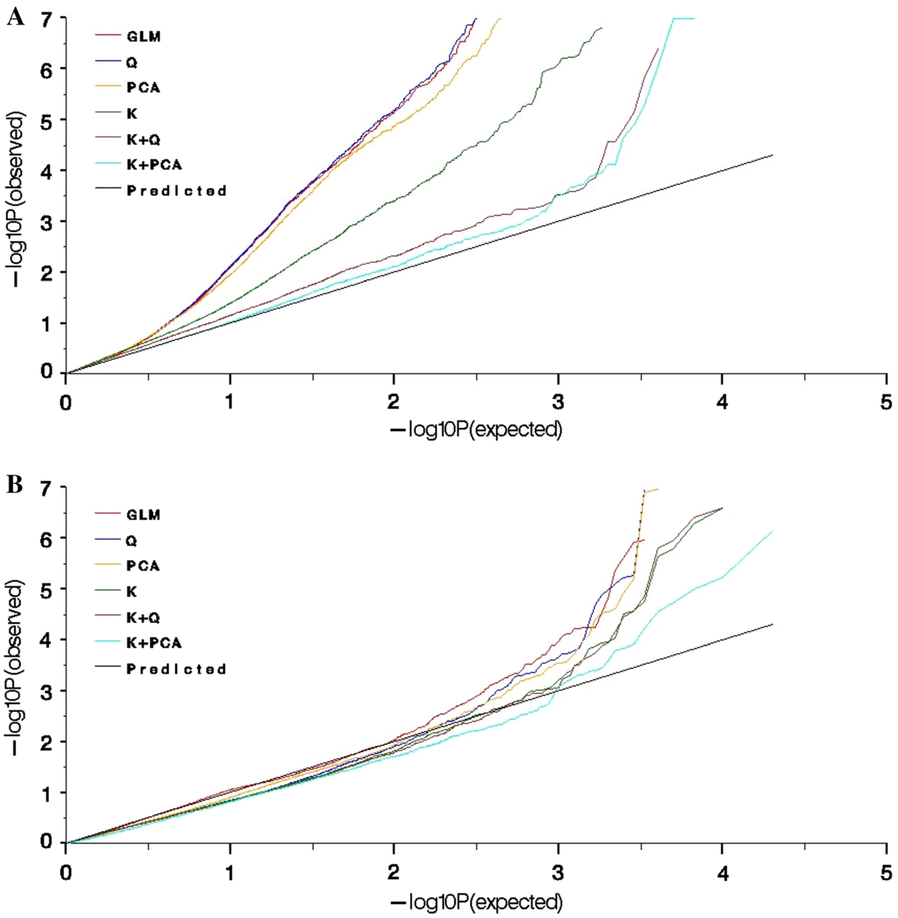

Fig. 4 Q–Q plots from six statistical models. **A** Q–Q plot for RRL; **B** Q–Q plot for RDW

Table 4 Associations loci. of SNP sites

Trait	Marker	Chr	Position (Kb)	*P* value	− lg (*P*)	Contribution (%)
RRL	Bn-A03-p28043216	chrA03	26,511,636	8.84E−07	6.053734463	20.456
	Bn-A10-p11601681	chrA07	2,573,086	2.24E−08	7.64889974	26.102
	Bn-A09-p9030563	chrA09	8,204,947	5.17E−06	5.286879227	15.806
	Bn-A10-p14327285	chrA10	14,264,189	1.04E−07	6.982173962	23.712
	Bn-scaff_23107_1-p158176	chrC05	168,088	5.19E−08	7.284832642	24.792
	Bn-scaff_20773_1-p622960	chrC06	5,117,105	2.20E−05	4.657478627	15.702
	Bn-A03-p17334423	chrC09	42,521,592	1.26E−05	4.900526544	14.566
	Bn-scaff_15650_1-p908638	chrC09	17,100,180	1.01E−07	6.996367163	23.763
RDW	Bn-A03-p8190847	chrA03	7,501,530	5.90E-06	5.229309959	16.87
	Bn-A04-p7776319	chrA04	9,100,305	9.83E−06	5.007362547	16.152
	Bn-A10-p15660472	chrA10	15,906,962	1.86E−05	4.729344451	15.257
	Bn-scaff_28179_1-p16904	chrC05	2,856,114	2.83E−05	4.548704917	14.679
	Bn-scaff_18520_1-p363396	chrC07	32,218,174	7.06E−07	6.150930866	17.83

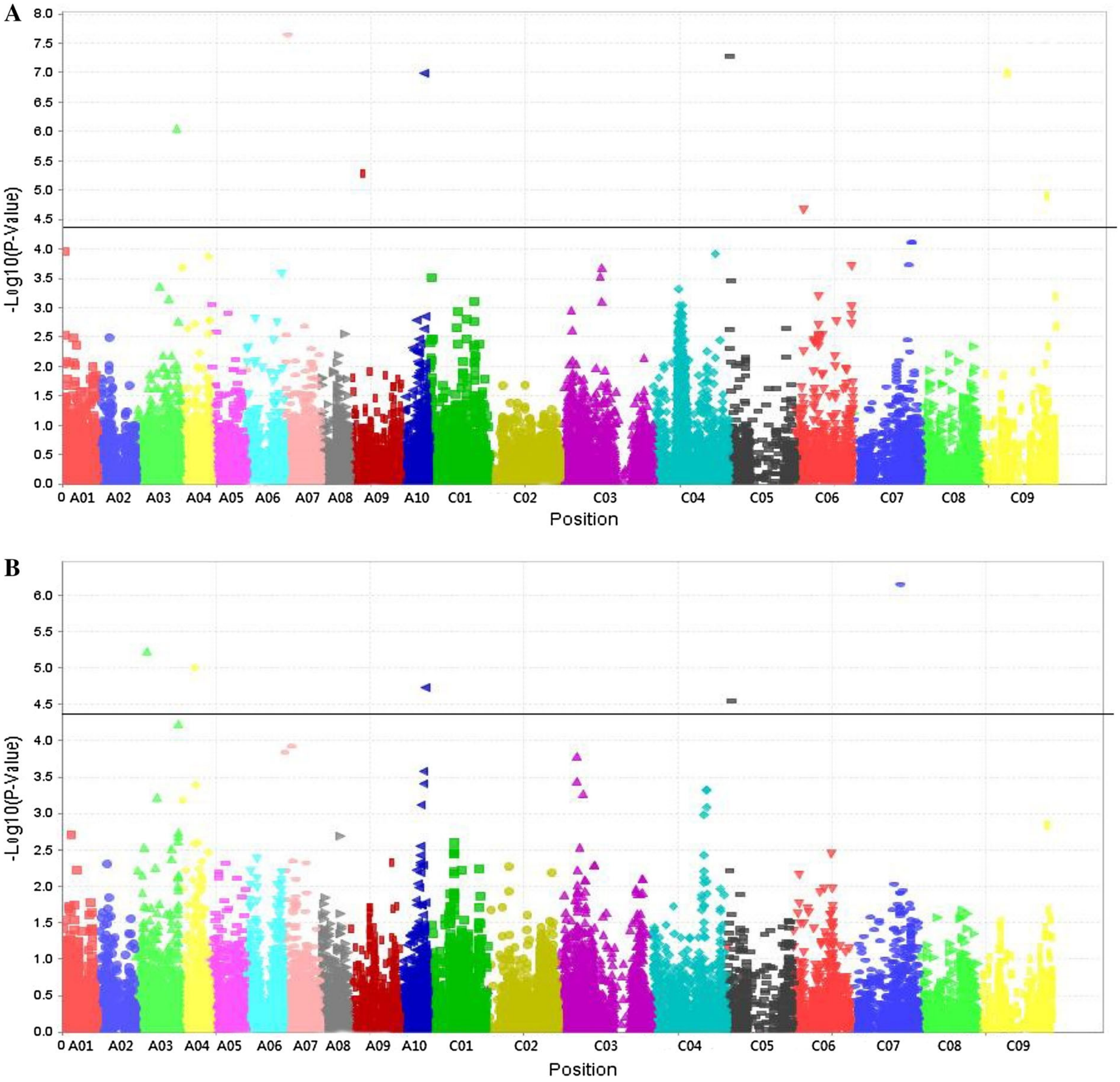

Fig. 5 Manhattan plots of − log10(*P*) versus chromosomal position. (− log10 *P* = 4.30). **a** Manhattan plot for RRL; **b** Manhattan plot for RDW

such as cold tolerance of maize (Huang et al. 2013a), salt and drought tolerance of sesame (Li et al. 2018), salt tolerance of rapeseed (He et al. 2017), as well as Al tolerance of rice (Tao et al. 2018) and barley (Cai et al. 2013). Many related SNP loci were successfully detected, and candidate genes were screened in these studies. To investigate the Al tolerance on seed germination, genome-wide association analysis (GWAS) of 19,949 SNPs with genome-wide coverage was used to identify the candidate genes related to germinate traits of rapeseed under Al stress. After doing a GWAS analysis of 169 rapeseed cultivars (lines), eight SNP loci were found to be associated with RRL and five SNP loci were correlated with RDW, respectively. These SNP markers, which were significantly associated with Al tolerance traits, could be selected as markers with higher contribution rate in breeding experiments. They can be used for the improvement of Al tolerance in rapeseed after further validation.

Table 5 Candidate genes for traits related to Al tolerance

Trait	*Brassica napus* genes	SNP locus	*Arabidopsis thaliana* genes	Gene interval (bp)	Gene name	References
RRL	BnaA03g51020D	26,511,636	AT1G73700	chrA03:26,472,257–26,474,531	MATE efflux family protein	Bock et al. (2006)
	BnaA03g51180D		AT4G30960	chrA03:26,561,750–26,563,043	SIP3	Sardar et al. (2017)
	BnaA03g51300D		AT4G31140	chrA03:26,633,249–26,635,425	O-Glycosyl hydrolases family 17 protein	Wang et al. (2007)
	BnaA07g02890D	2,573,086	AT2G17720	chrA07:2,508,180–2,509,784	P4H5	Velasquez et al. (2015)
	BnaA09g13940D	8,204,947	AT1G60980	chrA09:7,950,968–7,952,898	GA20OX4	Rieu et al. (2008b)
	BnaA09g13960D		AT1G60960	chrA09:7,961,803–7,962,996	IRT3	Zheng et al. (2018b)
	BnaA09g14730D		AT1G58340	chrA09:8,538,775–8,540,644	ABS4	Wang et al. (2015)
	BnaA10g19920D	14,264,189	AT5G13420	chrA10:14,002,070–14,004,200	TRA2	Ditt et al. (2006)
	BnaA10g20000D		AT5G13290	chrA10:14,053,091–14,054,368	CRN	Anne et al. (2018)
	BnaA10g20060D		AT5G13220	chrA10:14,087,599–14,090,438	JAZ10	Wang et al. (2018)
	BnaA10g20370D		AT5G12330	chrA10:14,277,819–14,279,081	LRP1	Estornell et al. (2018)
	BnaC05g00520D	168,088	AT1G01490	chrC05:298,074–299,804	MAE1.5	Hanada et al. (2011)
	BnaC06g14170D	17,100,180	AT3G52880	chrC06:16,984,806–16,987,349	MDAR1	Sarry et al. (2006)
	BnaC06g14250D		AT2G17420	chrC06:17,045,842–17,047,182	NTRA	Cha et al. (2015)
	BnaC06g14440D		AT3G53180	chrC06:17,211,394–17,215,355	NODGS	Dixon et al. (2005)
	BnaC06g14550D		AT3G53280	chrC06:17,318,363–17,320,551	CYP71B5	Murgia et al. (2011)
	BnaC06g14670D		AT1G48130	chrC06:17,435,695–17,436,032	PER1	Khare et al. (2017)
	BnaC06g14750D		AT3G53480	chrC06:17,509,657–17,511,905	PDR9	Fourcroy et al. (2016)
	BnaC06g14810D		AT3G53530	chrC06:17,532,412–17,535,160	NAKR3	Luo et al. (2016)
	BnaC09g39410D	42,521,592	AT5G18030	chrC09:42,017,664–42,017,943	SAUR21	Spartz et al. (2012)
	BnaC09g39650D		AT5G18200	chrC09:42,297,993–42,299,244	UTP	McCoy et al. (2006)
	BnaC09g39660D		AT5G18190	chrC09:42,300,801–42,303,898	AEL4	Zheng et al. (2018a)
	BnaC09g39680D		AT5G18170	chrC09:42,312,906–42,314,960	GDH1	Marchi et al. (2014)
	BnaC09g39770D		AT5G18020	chrC09:42,344,676–42,345,009	SAUR20	Kim et al. (2007)

Table 5 continued

Trait	*Brassica napus* genes	SNP locus	*Arabidopsis thaliana* genes	Gene interval (bp)	Gene name	References
	BnaC09g40230D		AT5G17700	chrC09:42,745,412–42,749,037	MATE efflux family protein	Ascencio-Ibanez et al. (2008)
	BnaC09g40390D		AT5G17520	chrC09:42,862,006–42,864,227	MEX1	Malinova et al. (2014)
	BnaC09g40420D		AT5G17490	chrC09:42,871,946–42,873,524	RGL3	Wild et al. (2012)
	BnaC09g40450D		AT5G17460	chrC09:42,880,935–42,881,316	DFR1	Ren et al. (2018)
	BnaC09g40460D		AT5G17450	chrC09:42,882,412–42,883,948	HIPP21	Tehseen et al. (2010)
	BnaC09g40960D		AT5G16910	chrC09:43,175,641–43,179,852	CSLD2	Yoo et al. (2012)
	BnaC09g41020D		AT1G76680	chrC09:43,205,115–43,206,819	OPR1	Blanco et al. (2005)
RDW	BnaA03g15880D	7,501,530	AT2G34555	chrA03:7,353,235–7,354,958	ATGA2OX3	Rieu et al. (2008a)
	BnaA03g15910D		AT2G34600	chrA03:7,365,766–7,366,111	JAZ7	Meng et al. (2019)
	BnaA03g15940D		AT2G34680	chrA03:7,375,651–7,386,626	AIR9	Buschmann et al. (2006)
	BnaA03g16180D		AT2G35612	chrA03:7,534,121–7,534,382	Copper amine oxidase family protein	Ohyama et al. (2008)
	BnaA03g16340D		AT2G35840	chrA03:7,627,872–7,629,594	SPP3B	Sarry et al. (2006)
	BnaA04g10180D	9,100,305	AT1G27070	chrA04:8,988,750–8,989,058	PTST2	Hanada et al. (2011)
	BnaA04g10260D		AT5G40390	chrA04:9,043,551–9,046,923	SIP1	Gangl and Tenhaken (2016)
	BnaA04g10280D		AT5G40440	chrA04:9,055,429–9,057,682	MKK3	Bai and Matton (2018)
	BnaA04g10460D		AT5G40610	chrA04:9,171,900–9,173,884	GPDHP	Li-Beisson et al. (2010)
	BnaA10g23640D	15,906,962	AT5G07200	chrA10:15,577,200–15,578,627	GA20OX3	Yan et al. (2014)
	BnaA10g23750D		AT5G07300	chrA10:15,642,261–15,645,529	BON2	Yang et al. (2010)
	BnaA10g23840D		AT5G07390	chrA10:15,673,427–15,677,274	RBOHA	Kaya et al. (2019)
	BnaA10g23880D		AT5G07440	chrA10:15,687,974–15,689,649	GDH2	Marchi et al. (2014)
	BnaA10g23990D		AT5G61410	chrA10:15,758,920–15,760,478	RPE	Jost et al. (2005)
	BnaA10g25040D		AT5G05365	chrA10:16,211,410–16,212,391	Heavy metal transport/ detoxification superfamily protein	De Abreu-Neto et al. (2013)

Table 5 continued

Trait	*Brassica napus* genes	SNP locus	*Arabidopsis thaliana* genes	Gene interval (bp)	Gene name	References
	BnaC05g05550D	2,856,114	AT1G07890	chrC05:2,700,362–2,701,742	APX1	Jiang et al. (2016)
	BnaC05g05770D		AT1G08110	chrC05:2,848,423–2,850,152	GLYOXALASE I	Jain et al. (2018)
	BnaC05g05800D		AT1G08135	chrC05:2,862,921–2,865,584	CHX6B	Maser et al. (2001)
	BnaC05g05810D		AT1G08140	chrC05:2,866,834–2,869,597	CHX6A	Cellier et al. (2004)
	BnaC07g25610D	32,218,174	AT3G29160	chrC07:31,733,775–31,736,220	KIN11	Chan et al. (2017)
	BnaC07g25770D		AT3G29320	chrC07:31,779,690–31,784,185	PHS1	Malinova et al. (2014)
	BnaC07g25860D		AT3G29670	chrC07:31,829,757–31,831,128	PMAT2	Taguchi et al. (2010)
	BnaC07g26120D		AT3G30775	chrC07:32,141,043–32,142,757	ERD5	Ren et al. (2018)
	BnaC07g26140D		AT3G30842	chrC07:32,154,460–32,161,586	PDR10	Crouzet et al. (2006)
	BnaC07g26270D		AT5G47910	chrC07:32,229,948–32,234,394	RBOHD	Zhai et al. (2018)
	BnaC07g27050D		AT5G49130	chrC07:32,676,878–32,678,493	MATE efflux family protein	Suzuki et al. (2015)
	BnaC07g27090D		AT5G49190	chrC07:32,686,727–32,690,692	SUS2	Angeles-Núñez and Tiessen (2010)
	BnaC07g27100D		AT3G07820	chrC07:32,693,755–32,695,669	Pectin lyase-like superfamily protein	Kim et al. (2006)

Phenotypic data and Al toxicity

Aluminum toxicity is a considerable hindrance of crop production in acidic soils, which constitute about 50% of the world's potentially arable lands (Kochian et al. 2005). At pH values beneath 5, Al tends to be dissolved as Al^{3+} ions, which are quite toxic to plant roots and can further limit crop production (Kochian et al. 2015). Germination and root growth tests have been used to evaluate the effect of Al tolerance in crops (Marciano et al. 2010). In this study, the RRL and RDW were used to evaluate the tolerance of 169 rapeseed germplasm resources at the germination stage under Al stress. The results showed that the average value of RRL was 0.727 and that of RDW was 0.964 in 169 rape germplasm resources, respectively, which indicated root length were seriously inhibited than dry weight though both of them were decreased under aluminum stress. Generally, Al toxicity inhibits root elongation, further leading to roots stunting accompanied by reduced water and nutrients uptake (Kochian et al. 2004), and result in the reduction of dry weight by altering carbohydrate synthesis and metabolism (Yuriko et al. 2007). Recently, the root apex has also recommended to role in Al tolerance, which is related to serious changes in the root system, including cell differentiation in root tips and lateral roots, interfering with several enzymes, increasing cell wall inflexibility, modifying the structure and capacity of plasma membranes, and disrupting signal transduction pathways (Sade et al. 2016; Min et al. 2019). The phenomenon was described shoots might be delayed relative to the root damages, or even be an indirect response to Al toxicity since they are generally a consequence of the root inhibition (Kochian et al. 2005).

Potential candidate genes

As a very complex process, seed germination depends both on the external environmental factors and the inner biochemical mechanisms controlled by a complex network of diverse but functionally interrelated phytohormones (Shu et al. 2016). Different cations [e.g., Al^{3+}, $Al(OH)^{2+}$] exhibit different degrees of toxicity to root, and the morphology of aluminum depends on the pH degree around rhizosphere (Kihara et al. 2003). Two genes consisting of *BnaC05g05800D* and *BnaC05g05810D* were found on chromosome C05 in this study, they could play a role in alleviating Al toxicity by regulating the pH level. Similar result was reported, which identified a mutant, ALR-104, proving that the pH gradient on the root surface can produce tolerance in *Arabidopsis thaliana* (Degenhardt et al. 1998).

On the other hand, plant hormones play an important role in the defense system of plant stress. Of them, phytohormones including cytokinin, auxin, delay senescence while salicylic acid (SA), abscisic acid (ABA), ethylene (ETH), and jasmonic acid (JA) promote senescence. GA can promote plant growth and development. In this study, three genes encoding gibberellin oxidase were detected, which named *BnaA09g13940D* (*GA20OX4*), *BnaA10g23640D* (*GA20OX3*) and *BnaA03g15880D* (*GA2OX3*), respectively. Amongst these, GA20-oxidase (*GA20OX*) is involved in the synthesis of GA, which can catalyze GA12 and GA53 to synthesize GA1 and GA4 with activity (Giacomelli et al. 2013), and *GA20OX* deficiency reduces the amount of synthesis of bioactive GA. While GA2-oxidase (*GA2OX*) is involved in the catabolism of GA, which can inactivate the biologically active GAs, its precursor as well as other intermediates, and maintain the balance between the biologically active GAs and intermediates in plants (Wuddineh et al. 2015). Besides, the plant hormone auxin (IAA) regulated many aspects of plant growth and development, including stem elongation, lateral branching of roots and shoots, establishment of embryonic polarity (Chapman and Estelle 2009). These processes are controlled by auxin-mediated changes in cell division, expansion, and differentiation (Nemhauser et al. 2006). We detected *BnaC09g39410D* (SAUR21) and *BnaC09g39770D* (*SAUR20*), which associated with cell expansion of plants. Likewise, Arabidopsis seedlings, which expressed an artificial microRNA targeting multiple members of the SAUR19–24 subfamily, exhibited short hypocotyls and reduced leaf size, and led to root waving, increased hypocotyl elongation, larger leaf size, reduced phototropism, and impaired apical hook maintenance (Spartz et al. 2012).

During defense, phytohormone-mediated signaling [e.g., abscisic acid (ABA), ethylene (ETH), jasmonic acid (JA), salicylic acid (SA)] is critically important for increasing resistance to stress. *BnaA04g10280D* (*MKK3*) and *BnaC09g40420D* (*RGL3*) were identified to activate ABA and ETH signaling pathway, positively regulate the synthesis of ABA and ETH, and alleviate Al toxicity damage through the transduction pathway of JA and SA, as well as negatively regulating the signal of GA and seed germination. In Arabidopsis thaliana, *MKK3* is involved in defense against pathogens, drought tolerance, JA signal transduction, and ABA and auxin responses (Li et al. 2017; Danquah et al. 2015; Enders et al. 2017). Similarly, it was suggested that the over-expression of *OsMKK3* (*oe-MKK3*) increased levels of jasmonic acid (JA), and abscisic acid (ABA), and decreased SA levels in rice after biotic stress (Zhou et al. 2019). Our results and these studies confirmed that *MKK3* played a pivotal role in the signaling pathway and defense responses under abiotic and biotic stresses. It is proposed that *MKK3* mediated positive regulation of rapeseed resistance to aluminum toxicity by inducing phytohormone dynamics. Furthermore, *BnaA03g15910D* and *BnaA10g20060D* belonged to ZIM domain proteins of JA and participated in the plant endogenous growth regulation. Previous studies had shown that JAZ proteins inhibit JA response, and likely to induce systemic resistance by regulating and affecting the signal transduction pathway of JA (Melotto 2008).

Plants have evolved different kinds of strategies to deal with Al toxicity in acid soils. In summary, two major elementary sorts of Al resistance mechanisms are characterized: Al exclusion mechanisms, which means to prevent Al particles from entering the roots, and Al tolerance mechanisms, which prefer to detoxify internal Al in the symplast (Sade et al. 2016). One of the most well-documented mechanisms is secretion of Al-induced root organic acid anions, malate, citrate, and oxalate, to chelate Al apoplastically (Lou et al.

2020; Ryan 2001; Yang et al. 2019), thus protecting cell wall from Al binding. Multi drug and toxin compounds extrusion (MATE) and aluminium-activated malate transporter (ALMT) are two transporter families responsible for Al-activated malate and citrate secretion, respectively (Kochian et al. 2015). In this study, the MATE efflux family proteins were identified on chromosomes A03, C07, and C09, which were *BnaA03g51020D*, *BnaC07g27050D*, and *BnaC09g40230D*, respectively. Some studies were showing that the MATE family genes can promote citrate excretion into the rhizosphere to protect roots from Al toxicity (Magalhaes et al. 2007; Ryan et al. 2009). Meanwhile, there was another gene found called *BnaA09g14730D* (*ABS4*) belonging to MATE transporter. Wang et al. (2015) reported that *ABS4* encodes a second putative MATE family transporter, and Arabidopsis MATE family of transporter genes including ABS4 can regulate cell elongation, further supporting the notion of a close functional relationship between the plant endomembrane system and cell elongation. In addition, ABS4 was able to transport citric acid, and participate in some physiological processes (Liu et al. 2009), and contributed to multidrug resistance and play major determinants of aluminum (Al) tolerance in plants (Min et al. 2019). However, there were no gene mutations related to malic acid transport in this study though the first Al tolerance gene about organic acids found in previous studies was malic acid transporter 1 activated by Al (ALMT1) (Sasaki et al. 2004). It is inferred that there might be two reasons for this: (1) The GWAS results are related to the effect value of traits caused by gene expression. It is difficult to detect them in GWAS analysis if the genes express little in RRL and RDW at the germination stage. (2) The ideal test for GWAS is a single factor test, which means that the interference of other factors within the study could lead to false-negative results. Moreover, two ABC transporters including ABCG37/*PDR9* and ABCG38/*PDR10* were identified, which belonged to the gene family encoding pleiotropic drug resistance (Fourcroy et al. 2016; Smart 2002). Some ABC transporters were associated with Al resistance (Xu et al. 2019). Among them, ABCG37/*PDR9* contributed primarily highly oxygenated coumarins to root exudation in Arabidopsis (Strader and Bartel 2009), while ABCG38/*PDR10* was a plasma membrane protein that plays a role in maintaining the proper distribution and function of a subset of other membrane proteins (Rockwell et al. 2009).

Apart from the genes above mentioned, we also found some genes related to growth and antioxidant stress, including *GDH*, *RBOHD*, *P4H5*, *NODGS*, *CYP71B5*, *OPR1*, *PDR9* and *PDR10*, and so on. Specifically, Glutamate dehydrogenase (GDH) is an important enzyme in nitrogen (N) metabolism. And it served as a link between C and N metabolism, in its role of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia (Magadlela et al. 2019). Furthermore, *P4H5* and *NODGS* were related to root growth. Previous study certified that *P4H5* was pivotal for root hair tip growth (Velasquez et al. 2015) and downregulation of NODGS resulted in plants with a short main root, reduced meristematic activity and disrupted development of the root cap (Doskocilova et al. 2011). In addition, a cytochrome P450 family gene *CYP71B5* on chromosome C06, which function was involved in oxidation reduction and alleviate oxidative damage via monooxygenase activity, iron ion binding, oxygen binding (Murgia et al. 2011). And its catalytic metabolic pathways can produce some important secondary metabolites, which strengthen plants resistant to pests, diseases, and other stresses (Sappl et al. 2009). So, it can be inferred that the gene is involved in the biological detoxification after aluminum stress. Also, *RBOHD* is responsible for the ROS burst after pathogen-associated molecular patterns (PAMPs) perception (Lee et al. 2020), through the phosphorylation and ubiquitination of *RBOHD*, the species of reactive oxygen species in plant immune process are regulated. Multiple evidences suggested that most ROS signaling during abiotic and biotic stress relies on the activities of two partially redundant isoforms, *RBOHD* and *RBOHF* (Miller et al. 2009; Kwak et al. 2003), increased of *RBOHD* mRNA levels resulted in accelerated leaf senescence. *BnaC09g41020D* (*OPR1*) could play important roles in plant defense under Al toxicity. Analogously, a number of works have shown a close relationship between *OPR1* genes and some physiological processes. Previous study showed that an *OPR1* isolated from Triticum aestivum confers salinity tolerance by means of scavenging oxygen species and enhancing ABA signaling (Dong and Wang 2013). A similar effect was found for *OsOPR1* in response to JA, SA, and ethylene in rice (Agrawal et al. 2003). Although, proline, an oxygen-free radical scavenger, is accepted as an indicator of various stresses, but it is not involved in protecting plants against metal toxicity (Yilmaz et al. 2012). In this study it is difficult to point the exact role of proline under the Al toxicity, as in the work realized in pigeonpea (Bhamburdekar and Chavan 2011) and *Lactuca sativa* L. (Silva and Matos 2016).

Taken together, Aluminum (Al) is a major metal component in soils and is solubilized as phytotoxic ions (predominantly Al^{3+}) which inhibit plant growth under low-pH conditions. Although rapeseed can produce certain defense strategies include pH regulation, metabolic adjustments, signaling cascades, and expression of different genes related to aluminum resistance, and reduce the harm of aluminum toxicity to seed germination, but the phenotypic data analysis of RRL and RDW showed that seeds germination and tender seedling growth were still inhibited. Generally, it is recognized alterations in the germination process as well as in the growth of the root, stems and leaves systems. Also, it is verified alterations in the biochemical processes, like inhibition of enzyme activity, and leading to nutrient deficiency symptoms (Kochian et al. 2005; Inostroza-Blancheteau et al. 2011). The screening of candidate genes laid a foundation for the functional identification of specific candidate genes in the genetic engineering of aluminum tolerance in rape.

Conclusion

The GWAS analysis of Al toxicity tolerance in rapeseed was conducted using RRL and RDW. The results showed that 13 SNP loci were significantly associated with these two traits. Among them, 8 SNP loci were significantly associated with RRL and located on chromosomes A03, A07, A09, A10, C05, C06, and C09, respectively. Five SNP loci were significantly associated with RDW and located on chromosomes A03, A04, A10, C05, and C07, respectively. Subsequently, fifty-nine function-known candidate genes related to Al tolerance were identified in the LD interval of these SNP loci. Four of these genes were involved in the growth regulation of organic acid, ten were involved in growth-regulating substance, eleven were related to oxidative stress, and nineteen were involved in carbon and nitrogen metabolism. All the genes could be potentially related to the Al tolerance of rapeseed. Whether they can influence Al tolerance needs to be verified by molecular assisted breeding or transgenic technology, to introduce these functional genes into rapeseed. This study laid out a foundation for further functional verification of genes and cultivation of new Al-tolerant varieties.

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Compliance with ethical standards

Conflict of interest There were no conflict of interest in this manuscript.

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