Breeding Science 67: 213–220 (2017)
doi:10.1270/jsbbs.16161

Research Paper

Analysis of cold resistance and identification of SSR markers linked to cold resistance genes in *Brassica rapa* L.

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Currently, cold temperatures are one of the main factors threatening rapeseed production worldwide; thus, it is imperative to identify cold-resistant germplasm and to cultivate cold-resistant rapeseed varieties. In this study, the cold resistance of four *Brassica rapa* varieties was analyzed. The cold resistance of Longyou6 and Longyou7 was better than that of Tianyou2 and Tianyou4. Thus, an F_2 population derived from Longyou6 and Tianyou4 was used to study the correlation of cold resistance and physiological indexes. Our results showed that the degree of frost damage was related to the relative conductivity and MDA content (r1 = 0.558 and r2 = 0.447, respectively). In order to identify the markers related to cold resistance, 504 pairs of SSR (simple sequence repeats) primers were used to screen the two parents and F_2 population. Four and five SSR markers had highly significant positive correlation to relative conductivity and MDA, respectively. In addition, three of these SSR markers had a highly significant positive correlation to both of these two indexes. These three SSR markers were subsequently confirmed to be used to distinguish between cold-resistant and non-cold-resistant varieties. The results of this study will lay a solid foundation for the mapping of cold-resistant genes and molecular markers assisted selection for the cold-resistance.

Key Words: cold resistance, *Brassica rapa*, correlation, SSR markers.

Introduction

In recent years, damage due to cold temperatures in China has caused great harm to the agricultural industry. Chinese rapeseed suffered severe cold damage because of continuous low temperatures in winter, leading to heavy losses and aggravating China's edible oil gap in 2008 (Zhang *et al.* 2008). Therefore it is of importance to cultivate cold-resistant rapeseed varieties. *Brassica rapa* has a long history of domestication in China, and some varieties have a robust cold hardiness, attracting the attention of researchers. Recently, *B. rapa* cultivation for cold resistance achieved a major breakthrough, and some cold-tolerant *B. rapa* varieties have been approved for agricultural use by the Chinese government, including 'Longyou6' and 'Longyou7'. These varieties have been widely grown near 48° latitude; for example, in the Xinjiang province, where these varieties grow well and there is little loss of yield, even when the temperatures reach –32°C (Sun 2013). Thus, these *B. rapa* varieties are good sources from which to develop cold resistant cultivars in China.

To date, a few research studies on the cold resistance of *B. rapa* have been published (Liu *et al.* 2014, Sun *et al.* 2007, Zhu *et al.* 2007). However, most of these studies were focused primarily on the characteristics of ground shoots under artificial control conditions. Studies on the physiological and biochemical characteristics of the underground roots have not been widely reported. Numerous studies have shown that cold resistance is closely related to plant cell membranes, enzymes, and a physiologically active defense system (Deng and Chen 2001, Jia and Guan 2012, Pu and Sun 2010). The unsaturated fatty acid content of the plasma membrane is associated with cold resistance in plants; varieties with a higher unsaturated fatty acid content in the membrane have better cold resistance (Roughan 1985). Low temperatures promote the synthesis of membrane phospholipids (Sun *et al.* 2009). Cold-resistant varieties are particularly adept at increasing the synthesis of membrane phospholipids in response to low temperatures (Willemot 1975). Under normal temperature conditions, the reactive oxygen content in plants is at a low level that does not hinder

Communicated by Christophe Rothan
Received September 29, 2016. Accepted February 19, 2017.
First Published Online in J-STAGE on May 23, 2017.
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growth and development. However, when subjected to low temperature, the plants produce an increased amount of reactive oxygen contents, resulting in peroxidation of membrane phosphor lipid, structural rearrangements of plasma membrane enzymes, and subsequent changes in the catalytic function of the membrane proteins (Boyer and Westgate 2004).

Since cold resistance in plants is controlled by numerous complex factors, it is difficult to determine the contributing factors under normal weather conditions. Studies have shown that morphological and physiological indicators can be used to evaluate cold resistance in plants. For cowpea and other plants subjected to low temperature stress, the activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) initially increase and then decrease (Peng *et al.* 1994). When the plants are subjected to cold stress, they have the ability to remove reactive oxygen by regulating the activity of protective enzymes while also regulating the concentration of intracellular proline, soluble sugars, and soluble proteins to maintain the stability of the intracellular environment, and to reduce stress and injury to plants due to low temperatures (Bais *et al.* 2003). Some studies have also shown that the cold resistance of plants is correlated with proline accumulation, which participates in the maintenance of osmotic balance between protoplasts and the external environment, as well as stabilizes the structure of biological macromolecules and maintains the structural integrity of the membrane (Hou and Tang 1999). The soluble sugar content is positively correlated with cold resistance in most plants. With the decrease of temperature, the soluble sugar content increases gradually, and the soluble sugar content increases more in the cold-resistant varieties than in the non-cold resistant varieties. However, in some plants, there is no correlation between the soluble sugar content and cold resistance (Lindow and Arny 1978). Soluble protein has been proven to enhance the cold hardiness of plants, and increasing the soluble protein concentration can enhance the cells ability to retain moisture, improving cold resistance capability (Jiang *et al.* 2002). Several studies have shown that varieties with high malondialdehyde (MDA) content also have higher levels of membrane lipid peroxidation, resulting in lower cold resistance (Fechner *et al.* 1986, Lin *et al.* 2012). The relative conductivity is also considered to be an important indicator, having a significantly negative correlation with cold resistance in *B. napus* (Huang *et al.* 2014). Therefore, these physiological indicators are often used to indirectly evaluate cold resistance in plants.

In recent years, molecular biology techniques have been used to determine the genetic components of cold resistance, and some cold resistance genes have been cloned and studied. *RCI3*, a POD-activity related cold-inducible gene, was isolated from rice, which not only enhances low temperature tolerance in plants, but also enhances tolerance of moisture and salt stress (Llorente *et al.* 2002). At present, cold regulated (*COR*) genes have been isolated and identified from *Arabidopsis thaliana*, canola, wheat and other plants (Cui *et al.* 2003, Zhang *et al.* 2016, Zhong *et al.* 2006). The *COR* genes encode various functional proteins to resist cold stress and improve cold resistance (Li *et al.* 2004). In addition, studies have found that *LEA* (Late embryogenesis abundant) genes may also contribute to cold hardiness in plants (Battaglia *et al.* 2008, Hundertmark and Hincha 2008). In addition, transcription factors related to cold resistance have been identified, including *APETALA2* (*AP2*), *NAC*, *MYB*, *bZIP* and *WRKY* (Liao *et al.* 2008, Ishiguro and Nakamura 1994, Jofuku *et al.* 1994, Paz-Ares *et al.* 1987, Souer *et al.* 1996). *APETALA2* (*AP2*) was first cloned from *Arabidopsis*, encoding a protein containing two *AP2*/*ERF* domains. Expression of *AP2*/*EREBP* transcription factor gene may increase the cold resistance of plants (Jofuku *et al.* 1994). Subsequently, this gene was isolated from maize, wheat, tomato, soybean, rice and other plants (Chen *et al.* 2009, Egawa *et al.* 2006, Gu *et al.* 2002, Matsukura *et al.* 2010, Moose and Sisco 1996).

In contrast, few cold resistance genes have been isolated from rapeseed, and there are very few reports on genetics and gene mapping. One reason is that cold resistance is a very complex issue, and it is difficult to determine the extent of cold damage directly; thus, we must use other indexes to measure cold resistance indirectly. In addition, results from different studies are sometimes inconclusive; therefore we set out to determine which specific indexes are related to cold resistance. In this study, we measured morphological and physiological indexes in various temperature conditions, in order to study correlation between these indicators and cold resistance in *B. rapa* rapeseed. We also constructed a segregating population and developed molecular markers for cold resistance gene mapping and marker assisted selection (MAS).

Materials and Methods

Two cold resistant *B. rapa* varieties ('Longyou6' and 'Longyou7') and two cold susceptible *B. rapa* varieties ('Tianyou2' and 'Tianyou4'), were used in this study, kindly supplied by Gansu Agricultural University. In order to identify the markers linked to cold resistance genes, an F_2 population consisting of 136 individuals was derived by hybridizing Longyou6 with Tianyou4.

Hydroponic growth was used to cultivate the four parental varieties and the F_2 population outdoors until the temperature reached 5°C. At least 120 plants for each variety were then transferred to V9 matrix (Peat:soil:perlite = 2:1:1, pH 4.5–7, TianFeng Horticulture, Shandong, China), and outdoor cultivation was continued. For the next several weeks, the local low temperature was observed and recorded, and the plants with low vigor were eliminated. Whenever the low temperature was below 5°C for 3 consecutive days, the leaves and roots of 15 individuals from each variety were selected on the morning of the third day to measure physiological indexes, including malondialdehyde (MDA), peroxidase (POD), catalase (CAT), and relative conductivity

(for leaves only). In total, three replicates were assessed. The MDA content was measured by the 2-thiobarbituric acid (TBA) assay. The protocol was as follows: the samples were ground in 10% trichloroacetic acid and centrifuged at 12000 rpm for 10 min; the supernatant was boiled for 15 min; the absorbance was measured at 450 nm, 532 nm and 600 nm, respectively. The POD activity was determined by the guaiacol colorimetric assay. The protocol was as follows: the samples were ground in 0.05 mol/L phosphoric acid (pH 5.5), and centrifuged at 3000 rpm for 15 min; the mixed solution was added with the 2.9 ml 0.05 mol/L phosphoric acid, 1 ml 0.05 mol/L guaiacol (pH 5.5) and 1 ml 2% H_2O_2; the mixed solution was incubated at 37°C for 5 min, and then for absorbance was measured at 470 nm. The CAT activity was measured by monitoring the UV absorbance. The brief method was as follows: the samples were ground in phosphoric acid (pH 7.0), and centrifuged at 4000 rpm for 15 min; the reaction solution was prepared by mixing 0.2 ml supernatant in 1.5 ml phosphoric acid (pH 7.0) and 1 ml distilled water; the mixed solution was incubated at 25°C for 3 min. The absorbance at 240 nm was measured after 300 µl 0.1 mol/L H_2O_2 was added. The membrane permeability (RPP) was determined by the conductance method. First, the samples were cut into small pieces, and then soaked in distilled water for 4 h at room temperature, the solution conductivity was measured. After being boiled for 15 min, the conductivity of the solution was measured again. The relative conductivity was calculated according to the equation: relative conductivity = extravasation conductivity/boil conductivity. The detailed protocols for calculating these four indicators were described by Li *et al.* (Li 2000). The days on which the above tests were completed were Oct 25, 2012; Dec 18, 2012; Dec 27, 2012; Jan 31, 2013; and Mar 12, 2013, with recorded low temperatures of 5°C, 0°C, –5°C, 0°C, and 5°C, respectively.

On the first day of measurement (Oct 25, 2012; 5°C low temperature), 20 individuals for each variety were used to measure morphological indexes, including leaf number, leaf length, root length, root diameter, and leaf area size, and three replicates were assessed. The wet weight and dry weight of leaf and root mass were measured, and the ratio of wet and dry weight of leaf and root mass, respectively, were also determined. The formula was as follows: dry and wet mass ratio (%) = dry weight/fresh weight × 100%. After preparing the original data as an excel format, the SPSS11.0 software was used for statistical analyses for these data (SPSS Inc., Chicago, IL). *LSR* (Least significant range) test (Gai 2000) was used to analyze whether there were significant differences among the four varieties. According to the results of multiple comparisons, the cold resistance of these varieties can be determined.

Subordinate functions were developed to analyze and evaluate the contribution of each factor to cold resistance according to three formulas (Zhu *et al.* 2011). The subordinate value of each indicator was calculated using the formula: $U(Xj) = (Xj - Xmin)/(Xmax - Xmin)$, $j = 1, 2, \ldots n$. Where, Xj, Xmin and Xmax represent the mean value, minimum value and maximum value of indicator j, respectively. The contribution of each indicator was calculated according to the formula: $Wj = Pj / \sum_{j=1}^{n} Pj$, $j = 1, 2, \ldots n$. Where, Pj was the contribution of indicator j. The comprehensive evaluation value of the varieties was calculated according to the formula: $Di = \sum_{j=1}^{n} [U(Xj) \times Wj]$, $i = 1, 2, \ldots k$. Where, k was the number of varieties. In order to compare the cold resistance among the four *B. rapa* varieties, the comprehensive evaluation value of the four varieties were analyzed using *LSR* test, and the multiple comparison was conducted (Gai 2000).

The frost damage of the F_2 population derived from Tianyou4 and Longyou6 was investigated in February, 2013, using methods previously described (Liu 1985). 136 individual plants were assessed individually for frost damage using a 0–4 scale, where 0 = no frost damage; 1 = frost damage on several large leaves; 2 = frost damage in half of the leaves, with central leaves undamaged; 3 = frost damage on most of the leaves, with central leaves undamaged; 4 = frost damage on all leaves including central leaves. For statistical analysis, a frost index was calculated using the following formula: Frost index = (1 × S1 + 2 × S2 + 3 × S3 + 4 × S4)/(total number of plants × 4). S1, S2, S3 and S4 represent the number of plants classified into grades 1–4, respectively. The leaves of the F_2 population were only measured for relative conductivity and MDA content in 0°C conditions (on Jan 31, 2013). These two indexes were measured by previously described methods (Li 2000). The frost damage rating of each individual (0–4) and the obtained physiological index data (the relative conductivity and MDA content) in F_2 were prepared. The r test (Gai 2000) was used to analyze the correlation between cold resistance and the physiological indexes in F_2 by the SAS8.1 software (SAS Inc., USA).

Genomic DNA was extracted from young leaves of F_2 individuals at the seedling stage by the CTAB method (Doyle and Doyle 1990). Final DNA concentration was adjusted to 50 ng/ul, SSR technology was used to screen the F_2 population and the two parental populations, and SSR amplification was performed as described by Lowe *et al.* (2002). Sequences of all of the SSR markers were obtained from public sources deposited at http://ukcrop.net/perl/ace/search/BrassicaDB (Lowe *et al.* 2004). 504 pairs of SSR primers distributed across the whole genome of *B. napus* were initially used to screen the parents, Longyou6 and Tianyou4, after which the polymorphic SSR primers were used to amplify the F_2 population. The amplified bands were expressed as '1' or '0', and the r test (Gai 2000) was conducted to analyze the correlation between the SSR markers and the physiological indexes by the SAS8.1 software (SAS Inc., USA). The SSR markers that can show a highly significant positive correlation to both physiological indexes were used to screen strong cold-resistant (grade 0) and cold-susceptible (grade 4) plants in the F_2 population and some cold-resistant *B. rapa* lines to validate the markers.

Results

Comparison of morphological characteristics before winter

On Oct 25, 2012, the low temperature was below 5°C, which essentially stopped the growth of rapeseed. By comparing the morphological characteristics of four *B. rapa* varieties, we found that the leaf number and leaf length of Tianyou2 and Tianyou4 were higher than those of Longyou6 and Longyou7, and the difference was significant ($P < 0.05$). However, in comparing other morphological characteristics, including root length, root diameter, dry to wet mass ratio of leaves, and dry to wet mass ratio of roots, Longyou6 and Longyou7 values were higher than Tianyou2 and Tianyou4, and the difference was significant ($P < 0.05$) or extremely significant ($P < 0.01$). These results demonstrated that the underground portions of the cold resistance varieties, (i.e. roots), grew more rapidly before winter compared to the non-cold resistant varieties. Dry matter accumulation in cold varieties was greater than in non-cold resistant varieties (**Table 1**).

Evaluation of cold resistance

In order to investigate the cold resistance of the four parental varieties, subordinate functions were developed to analyze and evaluate the contribution of individual factors to cold resistance. When subjected to the same low temperature stress, the difference in comprehensive evaluation values between varieties was very large. In addition, aboveground and underground comprehensive evaluation values were also different within the same variety. The comprehensive evaluation values were larger in the varieties with stronger cold resistance. The aboveground and underground comprehensive evaluation values of Longyou6 and Longyou7 were larger than those of Tianyou2 and Tianyou4, and the differences were significant ($P < 0.05$) or extremely significant ($P < 0.01$), indicating that Longyou6 and Longyou7 are more cold resistant than Tianyou2 and Tianyou4 (**Table 2**). Therefore, Longyou6 was selected to construct an F_2 population with Tianyou4 in order to identify cold resistance genes in *B. rapa*.

Investigation of frost damage in F_2

In order to investigate frost damage in the F_2 population, 136 individual plants were analyzed. Among these, 14 plants were free of frost with a percentage of 10.30%. The percentages of grade 1–3 were 13.25%, 25.7% and 37.5%,

Table 1. Morphological indexes results and dry to wet mass ratio

Testing indexes	Tianyou2	Tianyou4	Longyou6	Longyou7
Leaf number	8 ± 1.00ab	9 ± 1.00a	7 ± 1.00b	7 ± 0.00b
Leaf length (cm)	16.30 ± 0.44a	15.70 ± 0.75a	13.15 ± 0.06b	11.85 ± 0.04c
Root length (cm)	13.90 ± 0.10c	13.20 ± 0.30d	15.70 ± 0.44b	16.50 ± 0.40a
Root diameter (cm)	1.50 ± 0.26b	1.40 ± 0.17b	2.40 ± 0.26a	2.10 ± 0.35a
Dry to wet mass ratio of leaf (%)	12.7 ± 0.35b	12.3 ± 0.53b	15.2 ± 0.36a	14.7 ± 0.56a
Dry to wet mass ratio of root (%)	17.1 ± 0.36c	16.9 ± 0.53c	20.7 ± 0.56a	19.0 ± 0.26b

Table 2. Comprehensive analysis of cold resistance using physiological indexes

Temperature	Cultivars	Leaf				Mean	Root			Mean
		Relative conductivity	MDA content	POD activity	CAT activity		MDA content	POD activity	CAT activity	
5°C	Tianyou2	0.26 ± 0.01b	0.49 ± 0.01b	1.00 ± 0.17a	0.20 ± 0.1b	0.49 ± 0.03b	0.00 ± 0.00d	1.00 ± 0.10a	0.19 ± 0.03b	0.40 ± 0.04b
	Tianyou4	0.00 ± 0.00b	0.00 ± 0.00c	0.43 ± 0.06b	0.00 ± 0.00c	0.11 ± 0.02c	0.35 ± 0.07c	0.73 ± 0.07b	0.00 ± 0.00b	0.36 ± 0.04b
	Longyou6	1.00 ± 0.26a	0.82 ± 0.07a	0.00 ± 0.00c	1.00 ± 0.10a	0.70 ± 0.07a	1.00 ± 0.17a	0.22 ± 0.17c	0.74 ± 0.09a	0.65 ± 0.07a
	Longyou7	0.90 ± 0.20a	1.00 ± 0.26a	0.00 ± 0.00c	0.89 ± 0.12a	0.70 ± 0.07a	0.65 ± 0.09b	0.00 ± 0.00d	1.00 ± 0.26a	0.55 ± 0.12a
0°C	Tianyou2	0.00 ± 0.00c	0.24 ± 0.03b	0.00 ± 0.00d	0.14 ± 0.03c	0.09 ± 0.01c	0.36 ± 0.01c	0.36 ± 0.02c	0.23 ± 0.05c	0.32 ± 0.03b
	Tianyou4	0.37 ± 0.02b	0.00 ± 0.00c	0.18 ± 0.02c	0.00 ± 0.00d	0.14 ± 0.01c	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00c
	Longyou6	1.00 ± 0.26a	1.00 ± 0.10a	0.61 ± 0.05b	0.76 ± 0.05b	0.84 ± 0.06b	1.00 ± 0.17a	0.76 ± 0.03b	1.00 ± 0.19a	0.92 ± 0.06a
	Longyou7	0.88 ± 0.05a	0.99 ± 0.02a	1.00 ± 0.17a	1.00 ± 0.10a	0.97 ± 0.08a	0.78 ± 0.03b	1.00 ± 0.12a	0.78 ± 0.06b	0.85 ± 0.04a
–5°C	Tianyou2	0.13 ± 0.02d	0.47 ± 0.02c	0.09 ± 0.01d	0.08 ± 0.01c	0.19 ± 0.01c	0.00 ± 0.17c	0.13 ± 0.01c	0.14 ± 0.05c	0.09 ± 0.01c
	Tianyou4	0.37 ± 0.03c	0.00 ± 0.00d	0.18 ± 0.03c	0.00 ± 0.00d	0.14 ± 0.01d	0.30 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.10 ± 0.02c
	Longyou6	0.89 ± 0.02b	1.00 ± 0.11a	0.55 ± 0.04b	0.85 ± 0.03b	0.82 ± 0.05b	0.92 ± 0.05b	0.86 ± 0.08a	1.00 ± 0.19a	0.93 ± 0.03a
	Longyou7	1.00 ± 0.11a	0.80 ± 0.05b	1.00 ± 0.04a	1.00 ± 0.06a	0.95 ± 0.02a	1.00 ± 0.05a	1.00 ± 0.03b	0.65 ± 0.06b	0.88 ± 0.02b
0°C	Tianyou2	0.10 ± 0.02c	0.47 ± 0.04c	0.01 ± 0.02c	0.27 ± 0.03c	0.21 ± 0.00c	0.00 ± 0.00d	0.04 ± 0.01c	0.08 ± 0.02c	0.04 ± 0.01d
	Tianyou4	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00d	0.39 ± 0.04c	0.00 ± 0.00c	0.00 ± 0.00d	0.13 ± 001c
	Longyou6	0.76 ± 0.03b	1.00 ± 0.07a	0.38 ± 0.03b	1.00 ± 0.06a	0.79 ± 0.02b	1.00 ± 0.09a	0.59 ± 0.02b	1.00 ± 0.03a	0.86 ± 0.04a
	Longyou7	1.00 ± 0.04a	0.73 ± 0.04b	1.00 ± 0.10a	0.93 ± 0.02b	0.92 ± 0.03a	0.77 ± 0.04b	1.00 ± 0.04a	0.67 ± 0.04b	0.82 ± 0.03b
5°C	Tianyou2	0.00 ± 0.00d	0.45 ± 0.03b	0.00 ± 0.00d	0.09 ± 0.03c	0.14 ± 0.01c	0.26 ± 0.04b	1.00 ± 0.01a	0.00 ± 0.00c	0.42 ± 0.02b
	Tianyou4	0.23 ± 0.03c	0.00 ± 0.00c	0.30 ± 0.03c	0.00 ± 0.00d	0.13 ± 0.01c	0.00 ± 0.00d	0.42 ± 0.03b	0.01 ± 0.00c	0.14 ± 0.01c
	Longyou6	0.69 ± 0.02b	0.97 ± 0.02a	0.54 ± 0.03b	1.00 ± 0.09a	0.80 ± 0.01b	0.17 ± 0.01c	0.00 ± 0.00d	1.00 ± 0.10a	0.39 ± 0.04b
	Longyou7	1.00 ± 0.05a	1.00 ± 0.03a	1.00 ± 0.07a	0.85 ± 0.03b	0.96 ± 0.01a	1.00 ± 0.08a	0.35 ± 0.04c	0.61 ± 0.04b	0.65 ± 0.04a

The data for Relative conductivity, MDA, POD, and CAT are subordinate value of each indicator. Mean is comprehensive evaluation value of each cultivar.

respectively. The remaining plants were classified as grade 4 with a percentage of 13.25%, though most of these plants were unviable at the time of analysis. The average frost index of the F_2 population was 2.30.

Analysis of relative conductivity and MDA content

The relative conductivity and the MDA content of each individual plant in the F_2 population was measured. The maximum value of relative conductivity was 24.84%, and the minimum was 4.76% (average = 15.35%). The maximum MDA content was 8.52 μmol/gFW, the minimum was 0.75 μmol/gFW, and the average was 3.76 μmol/gFW (**Table 3**). These two indexes displayed a normal distribution, respectively (**Figs. 1**, **2**), showing that they were quantitative traits.

Correlation analysis of cold resistance and physiological indexes

SAS8.1 software was used to analyze the correlation between cold resistance and physiological indexes in F_2. Our results showed that the degree of frost damage was significantly correlated with relative conductivity and MDA content, with correlation coefficients of 0.558 and 0.447 ($r_{0.01,150} = 0.208$), respectively. The relative conductivity and MDA content were also significantly correlated with a correlation coefficient of 0.833 (**Table 4**). Therefore, we determined that relative conductivity and MDA content can be used to analyze the cold resistance of plants in our study.

SSR markers related to cold resistance

A total of 504 pairs of primers were used to screen the two parental populations, Longyou6 and Tianyou4. Of these, 97 primers showed polymorphism between the two parental lines, with a percentage of 9%. These polymorphic primers were then used to screen the F_2 population, from which 48 polymorphic loci were identified. In order to study the relationship between these loci and relative conductivity and MDA content, we analyzed the correlation of the SSR markers with relative conductivity and MDA content, respectively. The results showed that 10 SSR markers were correlated with relative conductivity and 11 SSR markers were correlated with MDA content. Four SSR markers (Na10-C03, BrGMS4511, BrGMS397, and BnGMS164) had a highly significant positive correlation to relative conductivity. Five SSR markers (Na10-C03, BrGMS4511, BrGMS397, BRAS011 and BnGMS67) also had a highly

Table 3. The relative conductity and MDA content in F_2 population and two parents

		Relative conductivity (%)	MDA content (μmol·g^{-1}·FW)
Parents	Longyou 6	10.92 ± 2.20	2.39 ± 0.94
	Tianyou 4	21.20 ± 2.50	4.73 ± 0.33
F_2	Maximum	24.84	9.02
	Minimum	5.37	0.95
	Mean	15.34	3.75

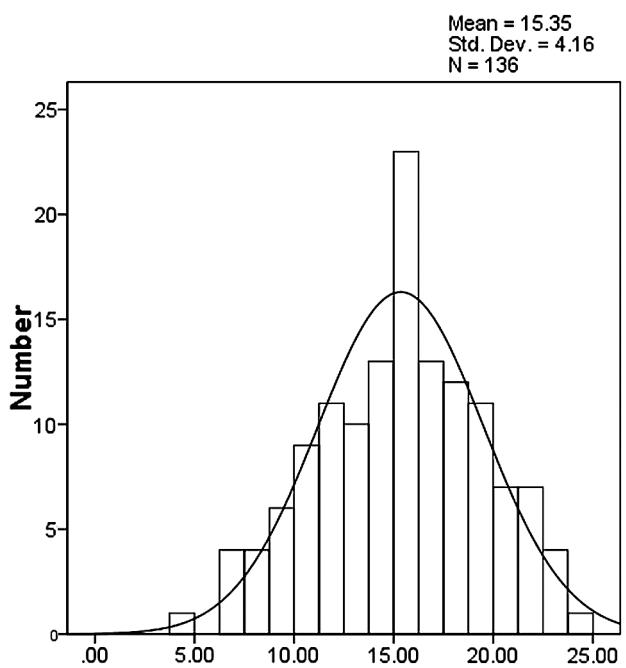

Fig. 1. Distribution of relative conductivity in F_2 population.

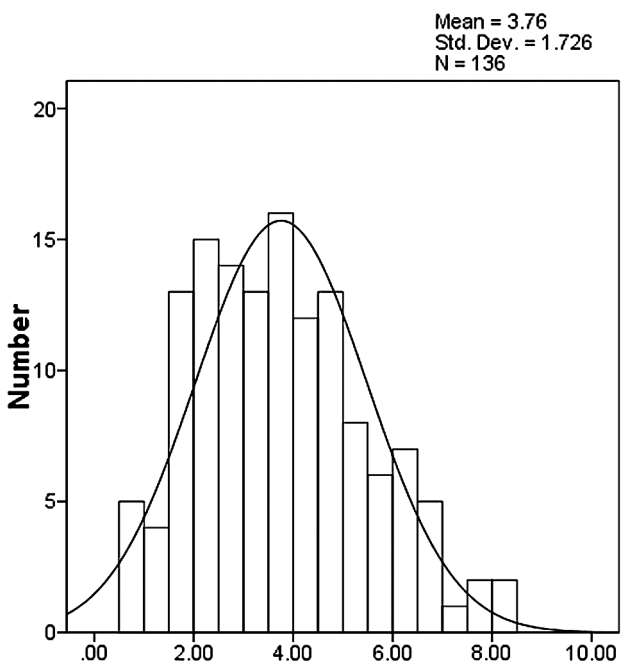

Fig. 2. Distribution of MDA content in F_2 population.

Table 4. Correlation between degree of frost damage and physiological indexes

	Degree of frost damage	Relative conductivity	MDA content
Degree of frost damage	–	0.558**	0.447**
Relative conductivity	0.558**	–	0.833**
MDA content	0.447**	0.833**	–

** stand for correlation at 0.01 levels. $r_{0.05(150)} = 0.159$, $r_{0.01(150)} = 0.208$.

Table 5. Correlation between SSR markers and physiological indexes

Primers name	Relative conductivity	MDA content	Primers number	Relative conductivity	MDA content
Na10-C03	0.236**	0.307**	BrGMS397	0.251**	0.226**
	0.149	0.192*	BrGMS3120	0.174*	0.181*
BRAS011	0.162*	0.271**	BrGMS171	0.205*	0.163*
BrGMS102	0.205*	0.193*	BnGMS67	0.091	0.316**
Ol13-G05	0.196*	0.152	BnGMS164	0.302**	0.172*
BrGMS4511	0.336**	0.307**	BrGMS579	0.340*	0.348*

* and ** stand for correlation at 0.05 and 0.01 levels, respectively. $r_{0.05(150)} = 0.159$, $r_{0.01(150)} = 0.208$.

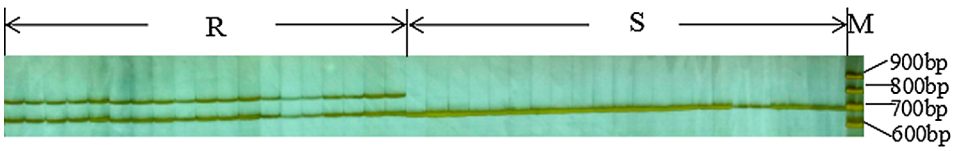

Fig. 3. Analysis of the PCR products obtained using BrGMS4511 on part of F_2 plants and *B.rapa* lines. R and S represented the resistant and susceptible individuals, respectively. M: 100 bp DNA ladder.

significant positive association with MDA content. Three SSR markers (Na10-C03, BrGMS4511 and BrGMS397) had a highly significant positive correlation to both of these two indexes (**Table 5**). These three markers were then used to screen the outlying plants in the F_2 population (14 plants at grade 0 and 18 plants at grade 4) and 9 *B. rapa* lines (5 plants at grade 0 and 4 plants at grade 4). These markers were able to effectively distinguish between the cold-resistant and non-cold resistant plants (**Fig. 3**); thus confirming that these three markers are correlated with cold resistance in our study.

Discussion

Frost frequently occurs throughout the world, and particularly in 2008, serious frost damage was experienced in most areas of China, leading to heavy losses in rapeseed production (Zhang *et al.* 2008). It is imperative to improve the cold resistance of rapeseed, and the most effective method is to develop cold-resistant varieties for rapeseed breeding. Most researchers agree that *B. rapa* has better cold resistance than other *Brassica* species (Li 2011, Yu and Park 2014), as this species includes many cold resistance genes. Longyou6 and Longyou7 are two *B. rapa* varieties cultivated in the northwest of China, where they thrive despite low temperatures that often reach –20°C, often lasting for 3 months. In order to evaluate the cold resistance of these two varieties, a variety of indicators were used. In addition, the subordinate function values of each physiological index were determined to evaluate the cold resistance of these plants and the results showed these two varieties were more cold resistant than other varieties, which was in consistent with previous studies (Gai *et al.* 2005, Pu and Sun 2010).

The cold resistance of crops is due to complex interacting factors, some of which are only activated in freezing conditions (Ma *et al.* 2016). Thus, it is difficult to evaluate these factors in normal growing conditions. An effective way to evaluate cold resistance is by using the physiological indexes of plants. In our research, the degree of frost damage of the F_2 population was found to be significantly correlated with relative conductivity and MDA content, respectively; therefore, these two indexes can be used to evaluate the cold resistance of plants indirectly. More interestingly, the Mendelian ratios and the Chi-square test of the frost damage rating showed that the plants with grade 0 and grade 1 accounted for nearly one-fourth of the individuals in the F_2. Therefore, the cold resistance of *B. rapa* in our study was likely to be controlled by a major gene, which needs further validation, because this result was based on the classification of the grade 1 plants as cold-resistant ones.

With the development of molecular biological techniques, it is possibility that identifying the molecular markers linked to relative conductivity or MDA content, which could be used for screening the cold resistant plants. Five and six SSR markers associated with relative conductivity and MDA content, respectively, were identified in our research. These markers will be helpful in the selection of cold resistant plants in the seedling stage or in normal (no frost) growing conditions. In this study, three SSR markers (Na10-C03, BrGMS4511 and BrGMS397) showed high accuracy in distinguishing between cold-resistant and non-cold resistant plants; thus, these markers can be used in MAS and gene mapping. In addition, it was found that these three markers were correlated with both relative conductivity and MDA content; therefore we propose that a single QTL locus on one chromosome controls these two traits (Na10-C03: A01; BrGMS397: A02; BrGMS4511: A03). So far, many cold-related genes have been annotated in *B. rapa*, including 1 on the chromosome A01, 2 on the chromosome A02, 6 on the chromosome A03 (https://www.ncbi.nlm.nih.gov/gene/). The SSR marker BrGMS397 (A02: 8.97 Mb) is located near a gene encoding a cold-inducible RNA-binding protein (A02: 4.97 Mb). The marker BrGMS4511 (A03:16.17 Mb) is also close to two cold-related genes (A03: 20.56 Mb and 21.74 Mb, respectively). Additionally, the results in the present study showed that the

relative conductivity and MDA content were inherited in quantitative traits. Therefore, it was inferred that the candidate genes of cold resistance in our study resist in these three regions. For other SSR markers identified in this study, they were found to be located on several chromosomes, indicating that several QTLs were responsible for these two traits. Future studies will be directed toward isolating the single QTL locus that controls both relative conductivity and MDA content. To achieve this goal, we plan to increase the density of molecular markers, and to use the QTL mapping method to identify the genes related to cold resistance found on chromosomes A01, A02, and A03.

Acknowledgements

The research was supported by National key research and development program (2016YFD0100200; 2016YFD0101300), Zhongying youth scholar program of Northwest A&F University and Shaanxi science and technology innovation program (2016KTCQ02-03).

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