GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Expression divergence of FRUITFULL homeologs enhanced pod shatter resistance in *Brassica napus*

P.F. Peng[1], Y.C. Li[1], D.S. Mei[1], J. Colasanti[2], L. Fu[1], J. Liu[1], Y.F. Chen[1] and Q. Hu[1]

[1]Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture, Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, Hubei, China
[2]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada

Corresponding author: Q. Hu
E-mail: huqiong@oilcrops.cn

Genet. Mol. Res. 14 (1): 871-885 (2015)
Received February 17, 2014
Accepted October 11, 2014
Published February 2, 2015
DOI http://dx.doi.org/10.4238/2015.February.2.11

ABSTRACT. To improve pod shatter resistance in the important oilseed crop *Brassica napus*, the phenotypic diversity of *B. napus* was tested using 80 *B. napus* varieties for pod shatter resistance by a random impact test. Among these varieties, R1-1 was identified as resistant, while R2, 8908B was susceptible to shatter. To understand the molecular basis for this phenotypic difference based on the candidate gene approach, *B. napus FRUITFULL* (*FUL*) homologs were identified and characterized. Two *FUL* loci in the A and C genomes of *B. napus* were identified. In the susceptible variety, both *BnaA.FUL* and *BnaC.FUL* were expressed in the same tissues. However, the expression level of *BnaC.FUL* differed in varieties with different pod shatter resistance. In the most resistant variety, R1-1, only *BnaA.FUL* was expressed, while *BnaC.FUL* was silenced. Therefore, the functional divergence and differing expression

of *BnaX.FUL* homeologs may significantly affect phenotypic variation, which is an important consequence of allopolyploid evolution. This expression level divergence may be useful for selecting pod shatter resistant lines through marker-assisted selection in *B. napus*-breeding programs.

Key words: Allopolyploid evolution; Expression divergence; *FUL* homeolog; Oilseed rape; Pod shattering

INTRODUCTION

The important oil crop *Brassica napus*, commonly known as oilseed rape, provides healthy edible oil for human consumption and may also be important as a biodiesel crop. At harvest, pod shattering can easily increase yield losses, particularly when seeds are harvested through mechanical means such as combines. Therefore, reducing pod shattering is a major objective in *B. napus*-breeding programs. However, little is known regarding pod shattering in oilseed rape because the pod shatter process is very complex, involving plant growth, silique development, hormone effects, and other processes (Child et al., 2003; Wang et al., 2007). In addition, the lack of oilseed rape germplasms resistant to pod shattering has limited this research (Wang et al., 2007).

The pods of *B. napus* contain 2 valves separated by the replum, which is in the center of the pod that extends along the 2 valves. Pod shattering involves the dehiscence zones (DZ), which are different from the valve edge cells and are located between the valve and the replum. In *Arabidopsis*, the DZ can be divided into 2 layers, a separation layer joined to the replum and a lignified layer joined to the valve (Lewis et al., 2006). DZ cells degrade and separate, which contributes to silique opening when the pods are mature.

Advances in molecular biology and genomics have enabled the development of oilseed rape lines resistant to pod shattering through genetic engineering or marker-assisted selection. Currently, with the development of whole-genome sequencing and functional gene identification methods in model plants, the candidate gene approach has been shown to be efficient for identifying key functional genes involved in specific processes (Salentijn et al., 2007). Oilseed rape has a high degree of genomic sequence identity to *Arabidopsis thaliana*, indicating its close relationship with this model plant species. Therefore, *A. thaliana* can be used to formulate hypotheses regarding gene function in *B. napus*. Homologs of *A. thaliana* genes typically have similar functions in *B. napus* (Chai et al., 2010). As functional genes have been identified in *A. thaliana* (Bolle et al., 2011), the use of these genes to identify functional *B. napus* genes with agronomic importance has become increasingly feasible.

Studies of *A. thaliana* have identified a complex regulatory network involved in pod shattering, including identification of some transcription factors involved in the development of valve margins. For example, *INDEHISCENT*, which is expressed in the 2 layers, is required for the development of valve margins (Liljegren et al., 2004), and *ALCATRAZ* (*ALC*) is involved in the development of the separation layers (Rajani and Sundaresan, 2001). Two MADS-box genes, *SHATTERPROOF1* and *SHATTERPROOF2*, promote the expression of *INDEHISCENT* and *ALC*, facilitating the lignification of DZ cells (Liljegren et al., 2000). The valves and the replum failed to detach in the *shatterproof1 shatterproof2* double mutant. *FRUITFULL* (*FUL*) regulates cell elongation and differentiation of the valve and negatively

regulates the expression of the *SHPs* (Gu et al., 1998; Ferrandiz et al., 2000). Ectopic expression of *Arabidopsis FUL* in *Brassica juncea* prevented differentiation of lignified cells in the valve margins, leading to pod shatter resistance (Ostergaard et al., 2006). *FILAMENTOUS FLOWER*, *YABBY3*, and *JAGGED* can control the expression patterns of *FUL* and *SHP* in the valves and valve margin (Dinneny et al., 2005). Similar to *FUL*, *REPLUMLESS* also negatively regulates *SHP* expression (Roeder et al., 2003).

Cultivated *Brassica* species include 3 diploids, *Brassica rapa* (AA genomes), *Brassica nigra* (BB), and *Brassica oleracea* (CC), and 3 allopolyploids, *B. napus* (AACC), *B. juncea* (AABB), and *Brassica carinata* (BBCC). The 3 diploid species were derived from an ancient hexaploid ancestor with a primary genome similar to that of *A. thaliana* (Lysak et al., 2005). The 3 allopolyploids originated from spontaneous hybridization between 2 of the 3 diploids (U, 1935). Each of the 3 allopolyploids contains 2 sets of homologous chromosomes, which originated from the 2 diploid species. Since the genome of *Brassica* species underwent a triplication event as well as other rearrangements during evolution, their genomes are more complex and divergent than those of *A. thaliana* (Lukens et al., 2003; Town et al., 2006). During genomic evolution in the *Brassica* species, homologous genes may have changed in function or become pseudo-genes after the 2 related diploid genomes fused and genetically recombined (Udall and Wendel, 2006; Chen and Ni, 2006). Indeed, change and rearrangement of homologous chromosomes have been demonstrated in *B. napus* (Udall et al., 2004). In the 3 *Brassica* allopolyploid genomes, functional changes in some homeologous genes may have also occurred.

In *A. thaliana*, *FUL* regulates valve margin elongation of the silique and promotes cell expansion in the valve, inhibiting pod shattering (Ferrandiz et al., 2000; Liljegren et al., 2004). Plant *FUL* genes have been shown to be involved in pod shattering, including *FUL* in *Arabidopsis* and *MADSB* in *Sinapis alba* (Chandler et al., 2005; Ostergaard et al., 2006). Ectopic expression of the *S. alba MADSB* gene, which is homologous to *AtFUL*, improved pod shatter resistance in *B. napus* (Chandler et al., 2005). Ectopic expression of the *Arabidopsis FUL* gene in *B. juncea* also prevented pod shattering (Ostergaard et al., 2006). Homologs of *Arabidopsis FUL* may also play an important role in pod development in *B. napus*.

As an allopolyploid crop, *B. napus* contains the A and C genomes, originating from *B. rapa* (A) and *B. oleracea* (C). Functional analysis of homeologs from the A and C genomes is of considerable importance for molecular manipulation of oilseed rape. In this study, we identified and examined *FUL* homologs in *B. napus*, *B. rapa*, and *B. oleracea*. Based on sequence differences of *FUL* genes from the A and C genomes, primers that could be used to distinguish *FUL* homeologs were developed. Using these homeolog-specific primers, reverse transcription (RT)-polymerase chain reaction (PCR) analysis showed that expression of *FUL* from the A genome was higher in pod shatter-resistant varieties than in susceptible varieties. This expression level divergence may be applied to select pod shatter-resistant lines in oilseed rape-breeding programs.

MATERIAL AND METHODS

Plant materials

A total of 80 varieties of *B. napus* were evaluated for pod shatter resistance. These materials included available open-pollinated cultivars and hybrids, which were all collected

by the Rapeseed Heterosis Application group, Oil Crops Research Institute, and Chinese Academy of Agricultural Sciences (OCRI-CAAS, Wuhan, Hubei). *Brassica rapa* var. Shang-hai-qing, Xi-zhang-bai-cai 1, Xi-zhang-bai-cai 2, Xi-zhang-bai-cai 3, *B. oleracea* var. Ye-sheng-gan-lan, Hei-ye-ping-tou, Ping-xiang-pei-lan, and Xin-nong-ying-liu-hua-cai were kindly provided by Prof. Xiaoming Wu in Department of Rapeseed Germplasm of OCRI-CAAS.

Assessment of pod shatter resistance

To identify varieties with different pod shatter resistance levels, 80 varieties of *B. napus* were screened by random impact test using a modified protocol (Wei et al., 2010). At the fully mature stage, pods were harvested and hung indoors for 4 weeks to balance their water content. Twenty well-developed intact pods were placed in a cylindrical container that was 19 cm in diameter and 14 cm in height, together with 12 steel balls that were 13 mm in diameter. The container was shaken on a horizontal shaker at 280 rpm. The number of cracked pods was counted after each minute of shaking and repeated for up to 10 min. The broken pods were removed from the container after each counting. Five plants of each variety were tested and pods were collected randomly from each whole plant. The pod shatter resistance index was calculated using the following equation:

$$(\mathrm{SRI}) = 1 - \sum_{i=1}^{10} x_i \times (11 - i) / 200 \qquad \text{(Equation 1)}$$

where X_i is the number of broken pods at the i^{th} minute, $1 \leq i \leq 10$.

DNA extraction, RNA extraction, and cDNA synthesis

Genomic DNA was isolated from leaves by cetyltrimethylammonium bromide extraction (Doyle and Doyle, 1990). Total RNA was isolated from various plant organs using Trizol reagent following manufacturer instructions (Invitrogen, Carlsbad, CA, USA) and was treated with RNase-free DNaseI (Qiagen, Hilden, Germany). Total RNA (2 μg) was reverse transcribed into cDNA using the M-MLV RTase cDNA Synthesis Kit (Takara, Shiga, Japan).

Identification and characterization of *BnaX.FUL* cDNA and genomic DNA

The nucleotide sequence of *Arabidopsis AGL*8 (AT5G60910) was used as query sequence to search against the EST database of *B. napus* in GenBank through BLASTN. Three ESTs (GenBank #EE448759.1, #EV092733.1, #CD841921.1) were identified and then chosen as the template sequences for PCR primer design. A 695-bp homologous sequence was amplified from R2 silique cDNA using primers FUL-F-1 and FUL-R-1 (all primer sequences are listed in Table 1). To obtain the entire cDNA sequence, 5'- and 3'-rapid amplification of cDNA ends (RACE) was carried out. The primer for 5'-RACE was FUL-RACE-5 and for 3'-RACE

was FUL-RACE-3. RACE was performed according to manufacturer instructions (Smart RACE cDNA Amplification kit, Clontech, Mountain View, CA, USA) with silique cDNA. According to sequences obtained from RACE, 2 primer pairs were designed to amplify the genomic DNA of the *FUL* gene from R1-1 and R2. The primers were FUL-F-2 and FUL-R-2, and FUL-F-3 and FUL-R-3. PCR was conducted using a 20-μL reaction system and consisted of 8.75 μL ddH_2O, 2 μL 10X LA PCR buffer, 4 μL 2.5 mM dNTPs, 2 μL $MgCl_2$ (25 mM), 1 μL of each 10 mM primer, 1 μL 100 mM DNA, and 0.25 μL Takara LA Taq (5 U/μL). The amplification conditions were as follows: pre-denaturation at 94°C for 3 min; 34 cycles for 45 s at 94°C, 45 s at 50°C, and 72°C for 3 min; finally, an 8-min extension at 72°C. All PCR products were separated by electrophoresis on 1.2% agarose gels and were purified using the DNA Rapid Purification Kit (Omega Bio-Teck, Norcross, GA, USA). Purified products were cloned into the PMD18-T vector (Takara) and transformed into *Escherichia coli* strain DH5α. A total of 12 single colonies for each PCR were picked for sequencing using the ABI 3730 (Applied Biosystems, Foster City, CA, USA).

Table 1. Primers used for PCR amplification in this study.

Prime name	Prime sequence (5'-3')	Prime purpose
FUL-F-1	AAAGAGACATGGGAAGGGGTA	*FUL* gene fragment PCR
FUL-R-1	GTTTGGTTCAATCAATGACGAT	
FUL-RACE-5	CAATGACGATGTACCGCCGTTCTC	*FUL* gene RACE
FUL-RACE-3	GAGAAGAACACGGGTCAGCAAGAAG	
FUL-F-2	TTATTTATGGGAAGGGGTAGGGT	*FUL* gene DNA PCR
FUL-R-2	TTGTTTGTCTGAATATAAATAGCGA	
FUL-F-3	ATGATCGCTATTTATATTCAGACAA	
FUL-R-3	CGTTCTTGACATTGTAATTCCGT	
MQ-FUL-F	CGCGGATCCATGGGAAGGGGTAGGGT	*FUL* gene CDS PCR
MQ-FUL-R	CCGCGGAATTCTCATACTTAGGTAATTTTACTCATT	
BrFUL-600-F	GCCCAGTACTGCGTAATCG	A genome special primer
BoFUL-600-F	GCCCAGTACTGCGTAATCT	C genome special primer
FUL-UTR-R	GTTCTTGACATTGTAATTCCGT	
BnActin-F	TCTGGCATCACACTTTCTACAACGAGC	Control
BnActin-R	CAGGGAACATGGTCGAACCACC	

Cloning of *FUL* homologs in *B. rapa* and *B. oleracea*

Using cDNA from a developing pod as template, the open reading frame of each FUL homolog was amplified using primers MQ-FUL-F and MQ-FUL-R. PCRs consisted of 10.75 μL ddH_2O, 2 μL 10X Ex Taq buffer, 2 μL 2.5 mM dNTPs, 2 μL $MgCl_2$ (25 mM), 1 μL of each 10 mM primer, 1 μL 100 mM cDNA, and 0.25 μL Takara Ex Taq (5 U/μL). The amplification conditions were: pre-denaturation at 94°C for 5 min; 34 cycles for 30 s at 94°C, 30 s at 62°C, 72°C for 1 min; 72°C for 8 min. After cloning and sequencing, sequence analysis was carried out using the DNAMAN software.

Homeolog-specific PCR primer design

Because the cDNAs of *BnaA.FUL* and *BnaC.FUL* shared a high level of sequence similarity, it was difficult to design primers for real-time quantitative RT-PCR. To perform semi-quantitative RT-PCR and allow comparison of expression levels of the homeologs, ho-

meolog-specific markers that could distinguish the *BnaA.FUL* and *BnaC.FUL* genes based on sequence divergence were developed. Four single nucleotide polymorphisms (SNPs) in the coding sequences (CDS) between *BnaA.FUL* and *BnaC.FUL* were identified by sequence comparison. Based on these SNPs, primers were designed using a modified single-nucleotide amplified polymorphism procedure (Drenkard et al., 2000). For this procedure, an SNP-specific base was introduced at the 3'-end of the forward primers. To improve the specificity and efficiency of genome-specific PCR, a "C" to "T" mismatched base was added at the third base to the 3'-end of the primers. When the A genome-specific primers were used to amplify *FUL* from the C genome, no amplification should occur because there were 2 mismatches in the 3'-end. However, when the *FUL* from the A genome was amplified using these primers, the amplification should be successful because there was only 1 base mismatch at the third base to the 3'-end. Therefore, it was possible to detect variation in polymorphic sites within *FUL* genes of *Brassica* A and C genomes by PCR. The genome-specific *FUL* gene primers were BrFUL-600-F and FUL-UTR-R for *BnaA.FUL* and BoFUL-600-F and FUL-UTR-R for *BnaC.FUL* (sequences listed in Table 1).

RT-PCR analysis of *BnaX.FUL*

Using homeolog-specific PCR primers BrFUL-600-F and FUL-UTR-R for *BnaA.FUL* and BoFUL-600-F and FUL-UTR-R, organ-specific expression of *BnaC.FUL* was detected. Various tissues including root, stem, leaf, bud, petal, developing silique, valve, stamen, and developing seed were collected at various plant developmental stages. The PCR conditions were as follows: pre-denaturation at 94°C for 3 min; 34 cycles for 30 s at 94°C, 30 s at 53°C, and 72°C for 1 min; finally, an 8-min extension at 72°C. For all RT-PCRs, the housekeeping gene *BnActin* (*B. napus* actin, AF1118122), as an internal gene control, was amplified using primers BnActin-F and BnActin-R (Table 1).The agarose gel images were analyzed using the Bio-Rad Image Lab 3.0 software (Hercules, CA, USA).

RESULTS

Screening for varieties with different levels of pod shatter resistance

To examine the regulation of pod shatter resistance in *B. napus*, we first examined the pod shatter phenotype of many varieties and identified varieties that were very resistant or very susceptible to pod shattering. Analysis of 80 *B. napus* varieties showed that the SRI varied widely among varieties, ranging from 0.003-0.710, with an average value of 0.182 (**Table S1**). Sixty-four varieties belonged to the susceptible shatter category, with SRI values below 0.300, while 15 varieties showed medium resistance with SRI values from 0.300-0.500; these values were considered as intermediate shatter resistance (Figure 1). Only 1 variety, R1-1, showed an SRI over 0.500, and was considered to be in the high shatter-resistant category. Based on these results, 2 extreme varieties were selected for further study, the resistant variety R1-1, with an SRI of 0.710, and the susceptible variety R2, with an SRI of 0.020 (Figure 2).

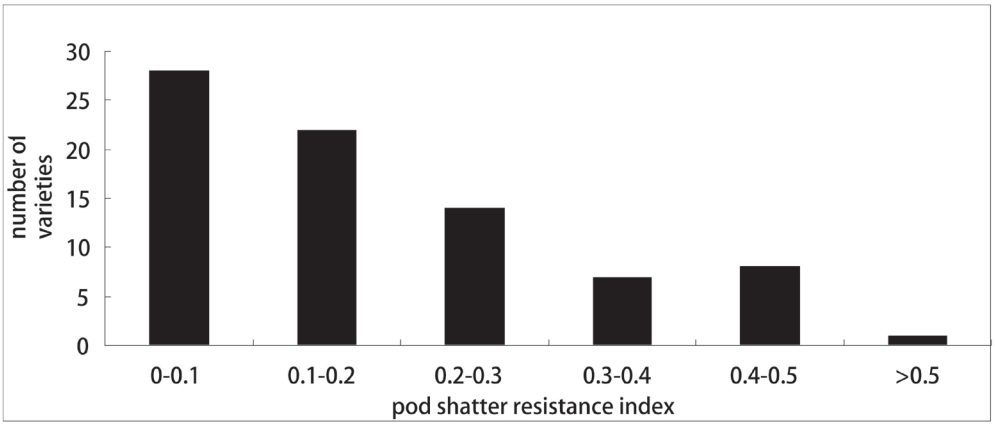

Figure 1. Distribution of pod shatter resistance indices in 80 varieties.

Figure 2. Pod shattering after random impact test using mature pods from resistant R1-1 and susceptible R2.

Cloning and sequence analysis of *FUL* homeologs in *Brassica*

With 5'- and 3'-RACE, 3 full-length cDNAs were identified in R1-1, whereas 7 full-length cDNAs were found in R2, including the 3 identified in R1-1. These cDNAs showed high sequence similarity to *AtFUL,* with identity over 93%, respectively, and thus were considered to be *B. napus FUL* homologs, referred to as *BnaX.FUL.x*. To assign the *BnaX.FUL.x* genes to *B. napus* genomes (A or C in the AACC allopolyploid), we next amplified the sequences of the *B. napus* ancestral species *B. rapa* (A genome) and *B. oleracea* (C genome). Sequence analysis of PCR products demonstrated that 3 *FUL* homeologs were present in *B. rapa* and 4 were present in *B. oleracea*. According to standard gene nomenclature (Ostergaard and King, 2008), the cDNAs identified in *B. napus* were named *BnaA.FUL.a*, *BnaA.FUL.b*, *BnaA.FUL.c* and *BnaC.FUL.a*, *BnaC.FUL.b*, *BnaC.FUL.c*, and *BnaC.FUL.d* based on their sequence similarity to the loci in *B. rapa* and *B. oleracea*, respectively. Sequence alignment

analysis showed that the cDNAs shared high sequence similarity. There was only 1-3 nucleotide difference among homologs within the same genome. The difference among homologs between genomes, however, was more diverse with a clear 7-bp deletion in *BnaA.FUL* at the 3'-end compared to *BnaC.FUL*.

The CDS of *FUL* homeologs in these 3 *Brassica* species were highly conserved. Sequence alignments showed that the sequence at the 5'-end was more conserved than the 3'-end. In the coding regions, mismatches, including transitions and 7 deletions, were observed, and all of these changes resulted in amino acid substitutions between *BnaA.FUL* and *BnaC.FUL*. DNA sequence analysis showed that the *BnaA.FUL* and *BnaC.FUL* in the *B. napus* genome both consisted of 7 introns and 8 exons (Figure 3). Sequence alignment of the complete genes revealed high similarity between the 2 *FUL* homeologs, with over 98% identity. Some mismatches were found in the introns, particularly in the first and sixth introns.

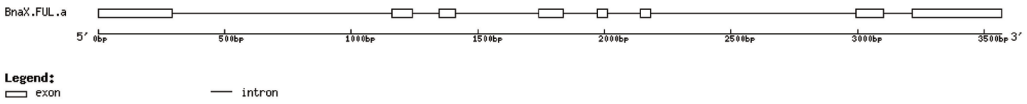

Figure 3. Structure of *BnaX.FUL* homeologs.

The deduced protein sequence of the *BnaC.FUL* protein contained 242 amino acids, while *BnaA.FUL* only contained 241 amino acids because of the 7-bp deletion of the CDS sequence. The 7-bp deletion led to a frameshift mutant, introducing premature termination for the CDS of amino acids in BnaA.FUL (Figure 4). Analysis using the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) showed that both of the predicted proteins contained a typical MADS-box and K-box, which were highly conserved in FUL homeologs. To compare differences in the protein sequences, multiple alignment of amino acid sequences revealed that the 7 FUL proteins shared 98% homology (Figure 4). In the MADS-box and K-box domain, the sequences were highly conserved. A homology tree revealed that the amino acid sequence from the same genome was more closely related to each other and clustered together, while BnaA.FUL.c was more closely related to BnaC.FUL than BnaA.FUL.a and BnaA.FUL.b (Figure 5).

Development of homeolog-specific markers for *BnaX.FUL* genes

The specificity of homeolog-specific primers for performing semi-quantitative RT-PCR was confirmed using genomic DNA of *B. napus*, *B. rapa*, and *B. oleracea* as templates. When the A genome-specific primers were used, the expected fragment was obtained from *B. napus* and *B. rapa*, whereas no amplified product was obtained from *B. oleracea* (Figure 6). Similarly, using the C genome-specific primers, 1 fragment was amplified from *B. napus* and *B. oleracea*, but no amplification occurred for *B. rapa*. Therefore, these fragments are specific markers for *FUL* homeologs from A and C genomes and are suitable for expression analysis of different *FUL* homeologs in *B. napus*.

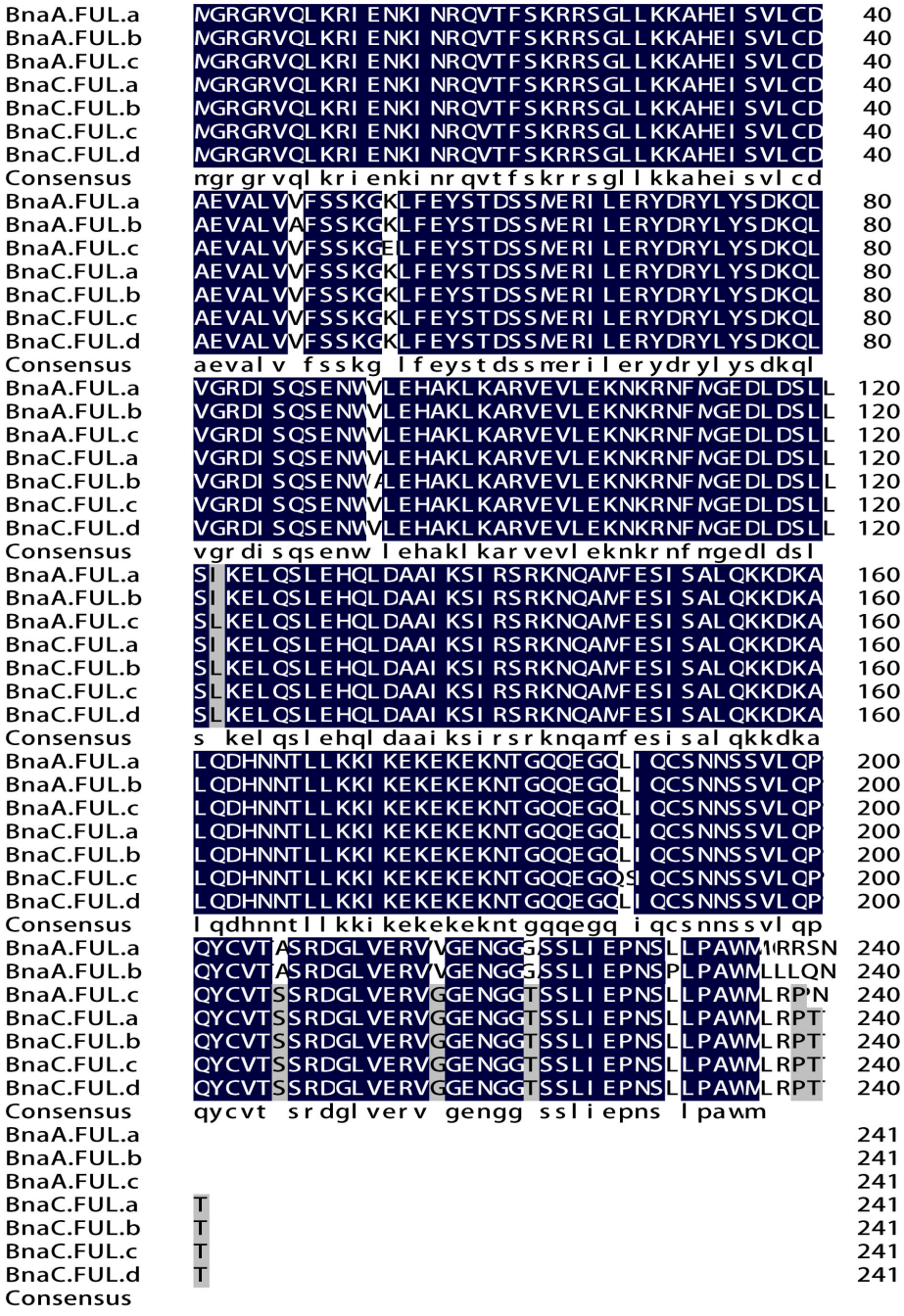

Figure 4. Sequence analysis of the 7 BnaX.FUL proteins. Dark shading revealed 100% sequence identity, white shading revealed above 75% sequence identity, and gray shading revealed above 50% sequence identity. Sequence alignment was carried out using the DNAMAN software.

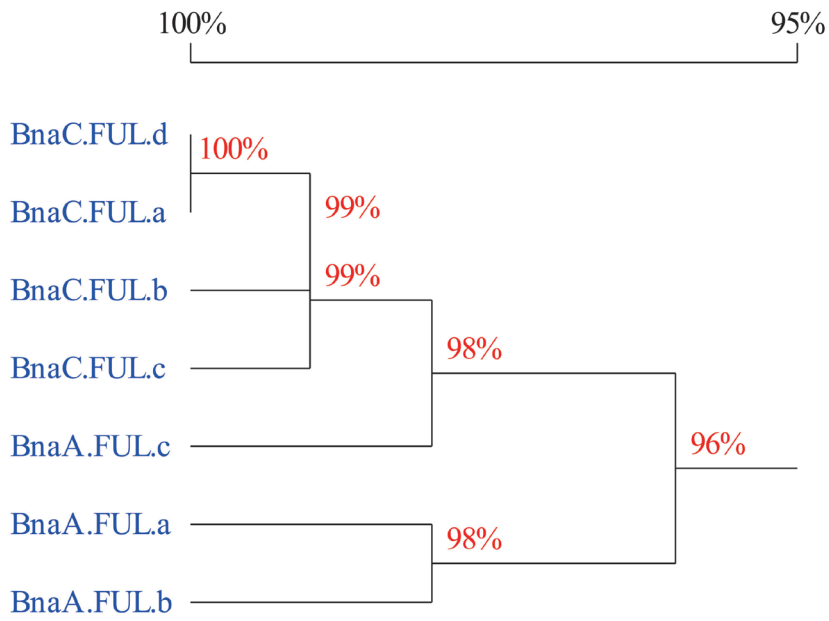

Figure 5. Homology tree of the 7 BnaX.FUL proteins. The bootstrap values are shown beneath each homology tree.

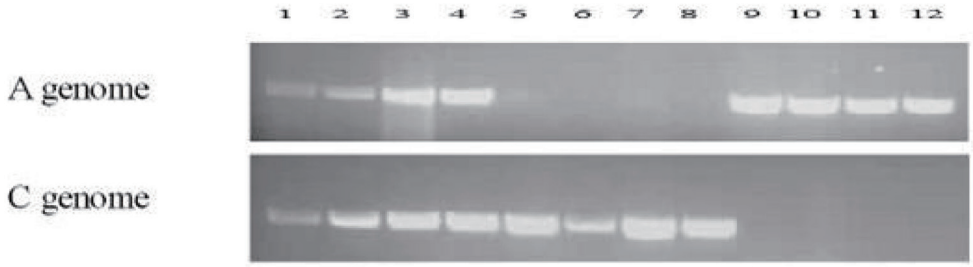

Figure 6. Amplification of *FUL* genes from different *Brassica* genomes using homeolog-specific primers. *Lanes 1-4*, *B. napus* R1, Zh11, R2, 8908B; *lanes 5-8*, *B. oleracea* Ye-sheng-gan-lan, Hei-ye-ping-tou, Ping-xiang-pei-lan, Xin-nong-ying-liu-hua-cai; *lanes 9-12*, *B. rapa* Shang-hai-qing, Xi-zhang-bai-cai 1, Xi-zhang-bai-cai 2, Xi-zhang-bai-cai 3.

Expression analysis of *BnaX.FUL*

To determine the expression pattern of *BnaX.FUL* in cultivars with different pod shatter resistance levels, semi-quantitative RT-PCR was performed using the homeolog-specific primers with cDNAs from R1-1 and R2 as templates. This showed that the *BnaX.FUL* genes were expressed in a wide range of organs, including the stem, leaf, bud, petal, developing silique, and valve, but not in the root, stamen, and seed. Both resistant and susceptible varieties showed the same pattern of tissue specificity (Figure 7), but 1 expression difference was detected between R1-1 and R2. In the susceptible variety R2, both *BnaA.FUL* and *BnaC.FUL* were expressed, with the highest expression in developing silique, whereas in the resistant variety R1-1, only *BnaA.FUL* was expressed and high expression was detected in

the young flower bud, developing pod, and valve. However, *BnaC.FUL* was not expressed in any tissue of R1-1 (Figure 7).

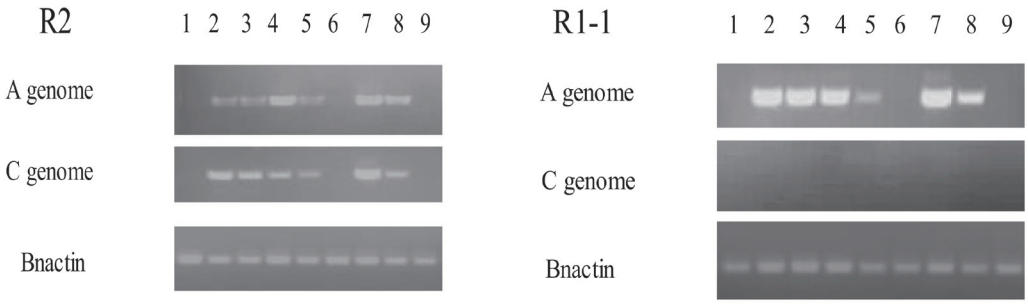

Figure 7. Expression of *BnaX.FUL* in R1-1 and R2 by RT-PCR. *Lane 1*, Root; *lane 2*, stem; *lane 3*, leaf; *lane 4*, young flower bud; *lane 5*, petal; *lane 6*, stamen; *lane 7*, developing pod; *lane 8*, valve; *lane 9*, developing seed.

To examine the correlation between the expression levels of *BnaX.FUL* and pod shatter resistance, we further analyzed 2 moderately resistant varieties Zh11 and R1, both with an SRI above 0.450 and 1 more susceptible variety 8908B (SRI = 0.003), using semi-quantitative RT-PCR with silique cDNA samples. The results clearly classified the varieties into 3 groups (Figure 8). In the first group, the highly resistant variety R1-1, only *BnaA.FUL* was expressed at a high level, whereas no *BnaC.FUL* expression was detected. In the second group, the moderately resistant varieties Zh11 and R1, both *BnaA.FUL* and *BnaC.FUL*, were expressed. In the third group, the susceptible varieties R2 and 8908B, both homeologs were expressed, but expression of *BnaA.FUL* was significantly lower than that in the other groups. The expression level of *BnaC.FUL* was the same across the varieties except for R1-1.

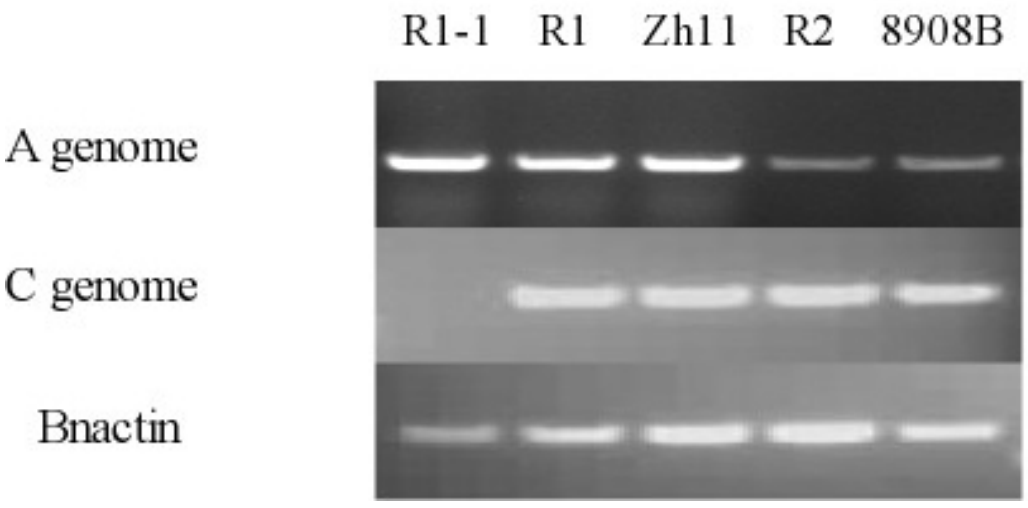

Figure 8. Semi-quantitative RT-PCR analysis of *BnaX.FUL* in siliques of *Brassica napus* varieties with different shatter resistance levels.

DISCUSSION

The genetic improvement of pod shatter resistance in *B. napus* has been limited by lack of resistant germplasms and a reliable assessment method (Wang et al., 2007). Here, we screened for pod shatter resistance in a wide range of *B. napus* varieties using an improved random impact test (Wei et al., 2010), and identified a highly resistant variety R1-1 after at least 3 years of testing (data not shown).

Previous studies identified a number of genes that positively or negatively regulate pod shattering, including *IND*, *ALC*, *SHP*, *REPLUMLESS*, and *FUL*, in *Arabidopsis*. Except for the *FUL* gene, these homolog genes showed no expression pattern variation. *FUL* was involved in valve margin elongation in *Arabidopsis* siliques (Liljegren et al., 2000). Ectopic expression of the *Arabidopsis FUL* gene in *B. juncea* prevented pod shattering and showed that valve margin specification was conserved between *Arabidopsis* and *Brassicas* (Ostergaard et al., 2006). In our study, to characterize the molecular differences associated with pod shatter resistance level, the *FUL* homeologs in *B. napus*, *B. rapa*, and *B. oleracea* were cloned and analyzed. The sequences of Bna*X.FUL* were highly conserved, as expected. Using the genome sequence of *B. rapa* (http://brassicadb.org) and GenBank database (http://ncbi.nlm.nih.gov) and a standard BLAST search, some paralogs with very high sequence identity were found. Because homeologs were derived from the same ancestral gene, they are typically highly conserved, as observed for many genes in polyploid species (Wang et al., 2009; Huang and Brûlé-Babel, 2010).

Our results indicate that expression level differences of *BnaX.FUL* may affect pod shatter resistance. Both *BnaA.FUL* and *BnaC.FUL* were expressed in the same organs, showing high levels of redundancy. However, the expression level of *BnaA.FUL* differed between resistant and susceptible varieties. In the pod shatter-resistant variety, *BnaA.FUL* expression was significantly higher than that in the susceptible varieties. Additionally, in the high pod shatter-resistant variety R1-1, only *BnaA.FUL* was expressed in the detected organs, whereas the *BnaC.FUL* transcript was not detected in any organs. To avoid errors in measuring the expression level because of the sample or PCR technique, different cycles of PCR, including 30 and 40 cycles, were performed, and the results were consistent (data not shown). Thus, expression divergence of *BnaA.FUL* play a role in pod shatter resistance for different varieties.

To overcome genome instability and reproductive degeneration, it has been suggested that polyploid species tend to acquire diploid-like characteristics through massive gene loss, genomic reorganization through silencing, and unequal expression of homolog genes (Adams et al., 2003; Chen, 2007). Alterations in gene expression upon allopolyploidization have been reported in many plant species, including cotton, wheat, *Arabidopsis*, *Senecio*, *Brassica*, and *Tragopogon* (Buggs et al., 2010). In *B. napus*, differential expression of homeologs was previously observed for the *ALC* (Hua et al., 2009) and *biotin carboxylase* (Li et al., 2011) genes. Xu et al. (2009) found that in resynthesized *B. napus*, the proportions of C-genome-specific gene silencing were significantly greater than those of A-genome-specific gene silencing. We found that *BnaC.FUL* expression was silenced, supported their results, but was different from the results for *BnaX.ALC* described by Hua et al. (2009) in terms of genome bias of gene silencing. There is no documentation on genome bias of gene silencing in *Brassicas*, and it is not clear whether gene silencing in *B. napus* is biased towards a specific genome as only a small number of genes was investigated. None of the above expression alterations of homologous genes, however, were correlated with phenotypic differences.

Alterations in the expression of homologous genes should lead to functional differences, as was recently shown for *BnGPAT*4 in *B. napus* (Chen et al., 2011). In this study, the absence of *BnaC.FUL* expression in the resistant variety did not occur in moderately resistant varieties. Instead, a difference in the expression levels of *BnaA.FUL* was observed across all varieties. Higher expression of *BnaA.FUL* was correlated with higher shatter resistance levels in the 5 tested varieties. Although in the highly resistant variety R1-1, silencing of *BnaC.FUL* appeared to play a role in enhanced resistance, the expression level of this homeolog did not differ in moderately resistant and susceptible varieties. It is more likely that the enhanced expression of *BnaA.FUL* was responsible for pod shatter resistance in resistant varieties rather than the depressed expression of *BnaC.FUL* in R1-1. In general, variations in pod shatter resistance in *B. napus* were limited; however, there was wide variation in *B. rapa* (Kadkol et al., 1985). Thus, the expression levels of *FUL* from the A genome that were correlated with pod shatter resistance may explain this observation. In allopolyploids, *cis*-regulatory elements and *trans*-action effects may alter the expression of homeologous genes. Functional divergence is often caused by changes in *cis*-regulatory elements (Wittkopp et al., 2004; Wang et al., 2006; Chen, 2007). We deduced that the *cis*-regulatory elements in the sequence of *BnaX.FUL* may be different, causing functional divergence of *BnaX.FUL*. The mechanisms behind the expression divergence of homeologous *FUL* loci and their effects on phenotypic variation require further investigation.

In most winter rapeseed planting areas, such as in China, the trend of combine harvesting of rapeseed will increase as labor resource shifting to cities from agricultural lands. Breeding rapeseed varieties with high pod shatter resistance is very important, but the number of pod shatter-resistant rapeseed germplasms is low. Thus, *BnaA.FUL* may be very useful for developing rapeseed varieties that are suitable for mechanical harvesting both through a transgenic approach and through marker-assistant selection using the expression level as a molecular marker.

In summary, 80 rapeseed varieties were screened for pod shatter resistance and some resistant varieties were identified. The homologous *FUL* genes in *B. napus*, *B. rapa*, and *B. oleracea* were cloned and characterized. SNP primers that could distinguish *FUL* gene loci from the different genomes of *B. napus* allowed for examination of the expression levels of *BnaA.FUL* and *BnaC.FUL* and revealed differences between resistant and susceptible varieties in *B. napus*. Importantly, expression levels of *BnaA.FUL.a* were correlated with the level of pod shatter resistance. The *BnaX.FUL* loci of different genomes showed sequence and function differentiation, which is an important consequence of allopolyploid evolution. The *BnaA.FUL* homeolog has great potential for the genetic improvement of oilseed rape, particularly for the development of lines suitable for mechanized harvesting.

ACKNOWLEDGMENTS

Research supported by the National Program on Key Basic Research Project ("973" Program, #2011CB109302), the National High-Tech R&D Program of China ("863" Program, #2012AA101107) of the Ministry of Science and Technology of China, the Earmarked Fund for China Agriculture Research System (CARS-13), and the Hubei Agricultural Science and Technology Innovation Center. We are grateful to Dr. Yancheng Wen for his instructions on pod shatter resistance assessment and Ms. Hongyu An for her assistance with the assessment.

Supplementary material

REFERENCES

Adams KL, Cronn R, Percifield R and Wendel JF (2003). Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proc. Natl. Acad. Sci. USA* 100: 4649-4654.

Bolle C, Schneider A and Leister D (2011). Perspectives on systematic analyses of gene function in *Arabidopsis thaliana*: new tools, topics and trends. *Curr. Genomics* 12: 1-14.

Buggs RJ, Elliott NM, Zhang L, Koh J, et al. (2010). Tissue-specific silencing of homoeologs in natural populations of the recent allopolyploid *Tragopogon mirus*. *New Phytol.* 186: 175-183.

Chai GH, Bai ZT, Wei F, King G J, et al. (2010). *Brassica GLABRA2* genes: analysis of function related to seed oil content and development of functional markers. *Theor. Appl. Genet*. 120: 1597-1610.

Chandler J, Corbesier L, Spielmann P, Dettendorfer J, et al. (2005). Modulating flowering time and prevention of pod shatter in oilseed rape. *Mol. Breed.* 15: 87-94.

Chen ZJ (2007). Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. *Annu. Rev. Plant Biol*. 58: 377-406.

Chen ZJ and Ni ZF (2006). Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. *Bioessays* 28: 240-252.

Chen X, Truksa M, Snyder CL, El-Mezawy A, et al. (2011). Three homologous genes encoding sn-glycerol-3-phosphate acyltransferase 4 exhibit different expression patterns and functional divergence in *Brassica napus*. *Plant Physiol*. 155: 851-865.

Child R, Summers JE, Babij J, Farrentand JW, et al. (2003). Increased resistance to pod shatter is associated with changes in the vascular structure in pods of a resynthesized *Brassica napus* line. *J. Exp. Bot*. 54: 1919-1930.

Dinneny JR, Weige D and Yanofsky MF (2005). A genetic framework for fruit patterning in *Arabidopsis thaliana*. *Development* 132: 4687-4696.

Doyle JJ and Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Drenkard E, Richter BG, Rozen S, Stutius LM, et al. (2000). A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in *Arabidopsis*. *Plant Physiol*. 124: 1483-1492.

Ferrandiz C, Liljegren SJ and Yanofsky MF (2000). Negative regulation of the *SHATTERPROOF* genes by *FRUITFULL* during *Arabidopsis* fruit development. *Science* 289: 54-78.

Gu Q, Ferrándiz C, Yanofsky MF and Martienssen R (1998). The *FRUITFULL* MADS-box gene mediates cell differentiation during *Arabidopsis* fruit development. *Development* 125: 1509-1517.

Hua SJ, Shamsi IH, Guo Y, Pak H, et al. (2009). Sequence, expression divergence and complementation of homologous *ALCATRAZ* loci in *Brassica napus*. *Planta* 230: 493-503.

Huang XQ and Brûlé-Babel A (2010). Development of genome-specific primers for homoeologous genes in allopolyploid species: the waxy and starch synthase II genes in allohexaploid wheat (*Triticum aestivum* L.) as examples. *BMC Res.* 3: 140.

Kadkol GP, Halloran GM and Macmillan RH (1985). Evaluation of *Brassica* genotypes for resistance to shatter. II.Variation in siliqua strength within and between accessions. *Euphytica* 34: 915-924.

Lewis MW, Leslie ME and Liljegren SJ (2006). Plant separation: 50 ways to leave your mother. *Curr. Opin. Plant Biol*. 9: 59-65.

Li ZG, Yin WB, Song LY, Chen YH, et al. (2011). Genes encoding the biotin carboxylase subunit of acetyl-CoA carboxylase from *Brassica napus* and parental species: cloning, expression patterns, and evolution. *Genome* 54: 202-211.

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, et al. (2000). *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. *Nature* 404: 766-770.

Liljegren SJ, Roeder AH, Kempin SA, Gremski K, et al. (2004). Control of fruit patterning in *Arabidopsis* by *INDEHISCENT*. *Cell* 116: 843-853.

Lukens L, Zou F, Lydiate D, Parkin I, et al. (2003). Comparison of a *Brassica oleracea* genetic map with the genome of *Arabidopsis thaliana*. *Genetics* 164: 359-372.

Lysak MA, Koch MA, Pecinka A and Schubert I (2005). Chromosome triplication found across the tribe *Brassiceae*. *Genome Res*. 15: 516-525.

Ostergaard L and King GJ (2008). Standardized gene nomenclature for the *Brassica* genus. *Plant Methods* 4: 10.

Ostergaard L, Kempin SA, Bies D, Klee HJ, et al. (2006). Pod shatter resistant *Brassica* fruit produced by ectopic expression of the *FRUITFULL* gene. *Plant Biotechnol. J*. 4: 45-51.

Rajani S and Sundaresan V (2001). The *Arabidopsis* myc/bHLH gene *ALCATRAZ* enables cell separation in fruit dehiscence. *Curr. Biol*. 11: 1914-1922.

Roeder AH, Ferrandiz C and Yanofsky MF (2003). The role of the *REPLUMLESS* homeodomain protein in patterning the *Arabidopsis* fruit. *Curr. Biol*. 13: 1630-1635.

Salentijn EMJ, Pereira A, Angenent GC, Linden CG, et al. (2007). Plant translational genomics: from model species to crops. *Mol. Breed*. 20: 1-13.

Town CD, Cheung F, Maiti R, Crabtree J, et al. (2006). Comparative genomics of *Brassica oleracea* and *Arabidopsis thaliana* reveal gene loss, fragmentation, and dispersal after polyploidy. *Plant Cell* 18: 1348-1359.

Udall JA, Quijada PA and Osborn TC (2004). Detection of chromosomal rearrangements derived from homologous recombination in four mapping populations of *Brassica napus* L. *Genetics* 169: 967-979.

Udall JA and Wendel JF (2006). Polyploidy and crop improvement. *Crop Sci*. 46: 3-13.

Wang J, Tian L, Lee HS and Chen ZJ (2006). Nonadditive regulation of *FRI* and *FLC* loci mediates flowering time variation in *Arabidopsis* allopolyploids. *Genetics* 173: 965-974.

Wang J, Long Y, Wu B, Liu J, et al. (2009). The evolution of *Brassica napus* FLOWERING LOCUS T paralogues in the context of inverted chromosomal duplication blocks. *BMC Evol. Biol*. 9: 271.

Wang R, Ripley VL and Rakow GR (2007). Pod shatter resistance evaluation in cultivars and breeding lines of *Brassica napus, Brassica juncea* and *Sinapis alba*. *Plant Breed*. 126: 588-595.

Wei WH, Li YC, Wang LJ, Liu SY, et al. (2010). Development of a novel *Sinapis arvensis* disomic addition line in *Brassica napus* containing the restorer gene for Nsa CMS and improved resistance to *Sclerotinia sclerotiorum* and pod shattering. *Theor. Appl. Genet*. 120: 1089-1097.

Wittkopp PJ, Haerum BK and Clark AG (2004). Evolutionary changes in cis and transgene regulation. *Nature* 430: 85-88.

Xu Y, Zhong L, Wu X, Fang X, et al. (2009). Rapid alterations of gene expression and cytosine methylation in newly synthesized *Brassica napus* allopolyploids. *Planta* 229: 471-483.