Pak. J. Bot., 48(2): 673-680, 2016.

CLONING AND EXPRESSION STUDY OF *BNALCR78* IN *BRASSICA NAPUS*

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Abstract

BnaLCR78 genes of three types of rape were cloned in rape (*Brassica napus*), and encoded protein structure was analyzed, the results showed that the protein had a conserved coding domain which was analogues among LCR family of Arabidopsis. The expression patterns of genes of three types of rape in varying tissues and in specific same tissues were analyzed using quantitative method. The results showed that their expression patterns differ from that of former research in *Brassica napus*, which may result from the difference of sampling time. We speculated that the gene might be involved in transpiration and transportation and distribution of nutrient, oil content in seed.

Key words: Cloning, BnaLCR78, *Brassica napus, Brassica campestris,* and *Brassica juncea*.

Introduction

As one of the major oil crops in the world, the improvement of rape quality has been one of the main goals of agricultural biotechnology. In generally, there are three types in rape, which are called *Brassica campestris*, *Brassica napus* and *Brassica juncea*, respectively. The yield of *Brassica napus* is the highest in three rape types. In the present stage, *Brassica napus* has occupied 95% of the total area of rape cultivation in china (Guan, 2006).

Two kinds of fatty acids which are called saturated fatty acid and unsaturated fatty acid including oleic acid are found in rape. The oil full of oleic acid can not easily be oxidized and smoked when heated to high temperature in the process of storage, refining and frying. High oleic acid oil can reduce the content of human body blood LDL cholesterol and prevent arteriosclerosis in the daily diet. So oleic acid is considered nutrition and health in the fatty acid (Grundy, 1986; Zhang *et al*., 2007; Guan & Li, 2008). In industry, oleic acid can be produced oleic acid ester and cosmetics (Liu *et al*., 2006). In recent years, improvement the oleic acid content has become the key of rapeseed quality improvement (Schierholt *et al*., 2000; Stoutjesdijk *et al*., 2000; Scarth & Tang, 2006).

By electronic cloning, no. 7 EST cloned fragment is obtained from 35 days after blossom seed specific expression library in *Brassica napus* "xiang you 15". The cloned fragment is named *BnaLCR78* because of high homology with one of a family gene member *LCR23* in Arabidopsis.

All the content of eicosenoic acid, erucic acid and eicosadienoic acid in *AtLCR23* mutant are changed obviously. Erucic acid and eicosadienoic acid are downstream products of arachidonic acid carbon chain elongation and desaturation pathways in the process of fatty acid synthesis. *LCR23* gene is speculated that involved in fatty acid metabolism in the form of serving as a molecular switch to adjust the synthesis of erucic acid and eicosadienoic acid. Earlier research has shown that the genome DNA sequence of *BnaLCR78* is obtained by electronic cloning and expression of the gene is studied by semi quantitative PCR (Peng, 2011). In the present study, both complete genome DNA sequence and cDNA sequence of *BnaLCR78* in three types of rape were obtained by RT-PCR method, and their encoding protein domain was analyzed. The tissue-specific expression patterns of *BnaLCR78* in three types of rape were preliminarily analyzed by fluorescence quantitative PCR.

Materials and Methods

Materials and reagents: The three types of rape seeds, including induced, high oleic acid content of rape (*Brassica napus*), the original control and low oleic acid content were grown in natural condition for 120 days to study the tissue-specific gene expression. Tissues of seedlings roots, stems, leaves, 27 d silique after blossom and 35 d silique after blossom were quickly put into centrifuge tubes, frozen in liquid nitrogen and kept at -80°C in refrigerator after sampling. Genomic DNA was extracted from leaf tissue using extraction method of Murry and Thompson (Murry & Thompson, 1980). RNA was extracted using TRIZOL reagent (Invitrogen, USA) and reverse-transcribed with a SuperScript III RT reagent kit (Invitrogen, USA) according to the manufacturer's instructions.

***BnaLCR78* clone:** Primers were designed based on the predicted three types rape *BnaLCR78* gene (Peng, 2011), using primer 5.0. The primers sequences amplified fragments of *BnaLCR78* were 5'-ATG GCG AAG CTA TCA TGT TCT-3'/5'-TCA ACA ATT CCA ATT ATA AGT AC-3'. PCR amplification was done using ES Taq DNA Polymerase (CWBIO, Beijing) with proofreading activity. The temperature cycles were: 4 min at 94°C, 40 s at 94°C, 40 s at 58°C, 30 s at 72°C for 35 cycles; and 7 min at 72°C. PCR products were purified by agarose gel recovery kit (TIANGEN, Beijing). The three cDNA frgments were ligated to pEASY-T1 (Transgen, Beijing), respectively. The fragments were sequenced by Invitrogen Co. Ltd (Invitrogen, Guangzhou), and sequencing results were analyzed using DNAMAN 6.0 software.

Conserved domain prediction: Using NCBI CDS bank (Conserved Domain Search, http:// www. ncbi. nlm. nih. gov/Structure/cdd), the conserved domain of *BnaLCR78* was predicted, and evolution tree analysis and amino acid sequence comparison with that of other species were conducted.

Real-time RT-PCR: For real-time RT-PCR, one pairs of primers (5'- TTGATTGTCGTCAAAACTGCTATG -3'/5'- TCTCCACCAGCTTTAACATCTTTAAC-3') and one probe (5'- ACAATGGAGTTGGAAAAT-3') were designed to amplify and detect fragment of the three types of *BnaLCR78*, respectively. One pair of primers (5'- CCTGGAATTGCTGACCGTATG -3'/5'- TGCGACCACCTTGATCTTCA-3') and one probe (5'- CAAAGAGATCACGGCGCTCGCAC-3') were designed to amplify and detect fragment of *β-actin*, which used as endogenous control for template standardization. After optimization of the parameters used for exponential amplification, the temperature cycle was designed as 40 cycles for three types of *BnaLCR78* and *β-actin*. The temperature protocol of gene and endogenous control were one cycle of 2 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C.

Results

Protein structure characteristic of BnaLCR78: *BnaLCR78* genes in three types of rape were cloned by RT-PCR, which have little differences in nucleotide level. For both of them, a complete ORF of 237 nucleotides were obtained which encoded 69 amino acid residues, respectively (Fig. 1a and 1b). Interestingly, ORF of the *BnaLCR78* gene of induced, high oleic acid content of rape has two forms. One was the normal, which encoded 69 amino acid residues. The other retained the intron and increase seven nucleotides, which contained 376 nucleotides and was similar to the sequences that obtained from genome DNA, can't be translated from TAG to TGA because of early termination (Fig. 2a and 2b). By using NCBI's Conserved Domain Database tools, the conserved domain of *BnaLCR78* was analyzed for in-depth exploration of its function. The result of our present study showed that *BnaLCR78* genes of three types of rapes had the one coding conserved domain, namely SLR1-BP, which had a high consistency to Arabidopsis (Figs. 3 and 4). The alignment in protein level and phylogenetic analysis showed that Arabidopsis LCR25 and Arabidopsis LCR26 had a consistency the highest of 79%, which had a consistency the consistency of 54% to rape aLCR78. The consistency between Arabidopsis LCR21 and LCR22, Arabidopsis LCR35 and LCR36 were74% and 70%, respectively, the consistency of the later two classes was 60%, which had a consistency of 56% to Arabidopsis LCR23. The consistency between Arabidopsis LCR23 and rape aLCR78 was only 50% and was the lowest (Fig. 5).

Expression of *BnaLCR78* in three types of rape in different tissues: The expression of *BnaLCR78* in different tissues of three types of rape grown 120 days in natural outside room condition were examined in order to study the gene's role in plants' development. The result indicated that the highest level of *BnaLCR78* in G type was in 35d silique after blossom, expression in stem was respectively 50% of the highest level in 35d silique after blossom tissue. Expressions in other tissues were low when compared to that of the expression in 35 d silique after blossom. The highest level of *BnaLCR78* in CK type was in root, expression in 35 d silique after blossom was respectively 25% of the highest level in root tissue. Expressions in other tissues were low when compared to that of the expression in root. The expression level of *BnaLCR78* in D type also reached the highest in 35 d silique after blossom. Expression in 27 d silique after blossom tissue was respectively 30% of the highest level in 35 d silique after blossom. Expressions in other tissues were low when compared to that of the expression in 35 d silique after blossom (Fig. 6).

Expression of *BnalCR78* in three types of rape in the same tissues: The expressions of *BnaLCR78* in different tissues of three types of rape were conducted. And results showed that expression levels in three types of rape in leaves and in 35 d silique after blossom were closely equal (Fig. 6). In order to distinguish the real difference among three types of rape, the expression of *BnaLCR78* in three types of rape in the same tissues were examined. The results showed that the expressions of three types of rape in root, stem and 27 d silique after blossom were closely conformed to the results of figure 6. Additionally, the expressions of three types of rape in leaves and 35 d silique after blossom were fallen short of the results of figure 6. The highest level of *BnaLCR78* in G type was in leaves, and expression levels in CK type and D type were closely equal, which were 90% of the expression in G type in leaves tissue. The highest level of *BnaLCR78* in G type was in 35 d silique after blossom, and expression levels in CK type and D type were closely equal, which were 1% of the expression in G type in 35 d silique after blossom tissue (Fig. 7).

Discussion

In this study, cDNA sequences of *BnaLCR78* in three types of rape were analyzed by PCR, and the results showed that there were little differences among them. Additionally, two forms of cDNA sequences of G type rape were analyzed by the same method, and the results showed that one form was normal that cut off the intron from genome DNA. The other form that retained the intron from genome DNA cannot be translated from ATG to TGA because of early termination. Previous research indicated that the mechanism of intron retention was found in many other species. In barley, an intron retaining K+ transporter may play a role in salt stress (Shahzad *et al*., 2015). Annotations of the alternatively spliced genes revealed that they represent diverse biological process and molecular functions, suggesting a fundamental role for alternative splicing in affecting the development and physiology of *S. bicolor* (Panahi *et al*., 2014). Not only intron retention was found in monocotyledonous plants, but also found in dicotyledonous plants. In Arabidopsis, comparing coordinates of introns of all annotated mRNAs from TAIR10, putative regulatory motifs in intron splicing were predicted based on feature extraction approach (Mao *et al*., 2014). A genome-wide analysis revealed that SKIP mediates the alternative splicing of many genes under salt stress conditions, and that most of the alternative splicing events in skip-1 involve intron retention, which generates a premature termination codon in the transcribed mRNA (Feng *et al*., 2015). Many alternative splicing events may have important, but uncharacterized, functions, are conserved between *Brassica* and *Arabidopsis*(Darracq & Adama, 2013).In *Prunus salicina Lindl*, *PsARF/XYL* gene is post-transcriptionally regulated by alternative splicing during development and that ethylene may be involved in this regulation (Di Santo *et al*., 2015). Additionally, intron retention was found in animals. Through study the constitutive transport element, all essential functional components for expression of mRNA with retained introns have been conserved from fish to man (Wang *et al*., 2015). Using high-coverage poly(A)(+) RNA-seq data, we observe that IR is surprisingly frequent in mammals, affecting transcripts from as many as three-quarter of multi-exonic genes (Braunschweig *et al*., 2014). Intron retention was also found in germ except to Eukaryotes. Through studied on *Aspergillus oryzae*, between splicing efficiency and the necessity of OcpG activity for obtaining a nitrogen source might be a correlation. Furthermore, OcpG intron retention might be affected by the secondary structures of intronic mRNA (Ishida *et al*., 2014). In *Neurospora crassa*, the intron-retained PRE-1 variant is predicted to lack 6 ubiquitination sites that may influence receptor function (Strandberg *et al*., 2013). Like these genes and transcription factors mentioned above, *BnaLCR78* was found in the form of intron retention suggested it may played a role in regulation the development and quality in rape.

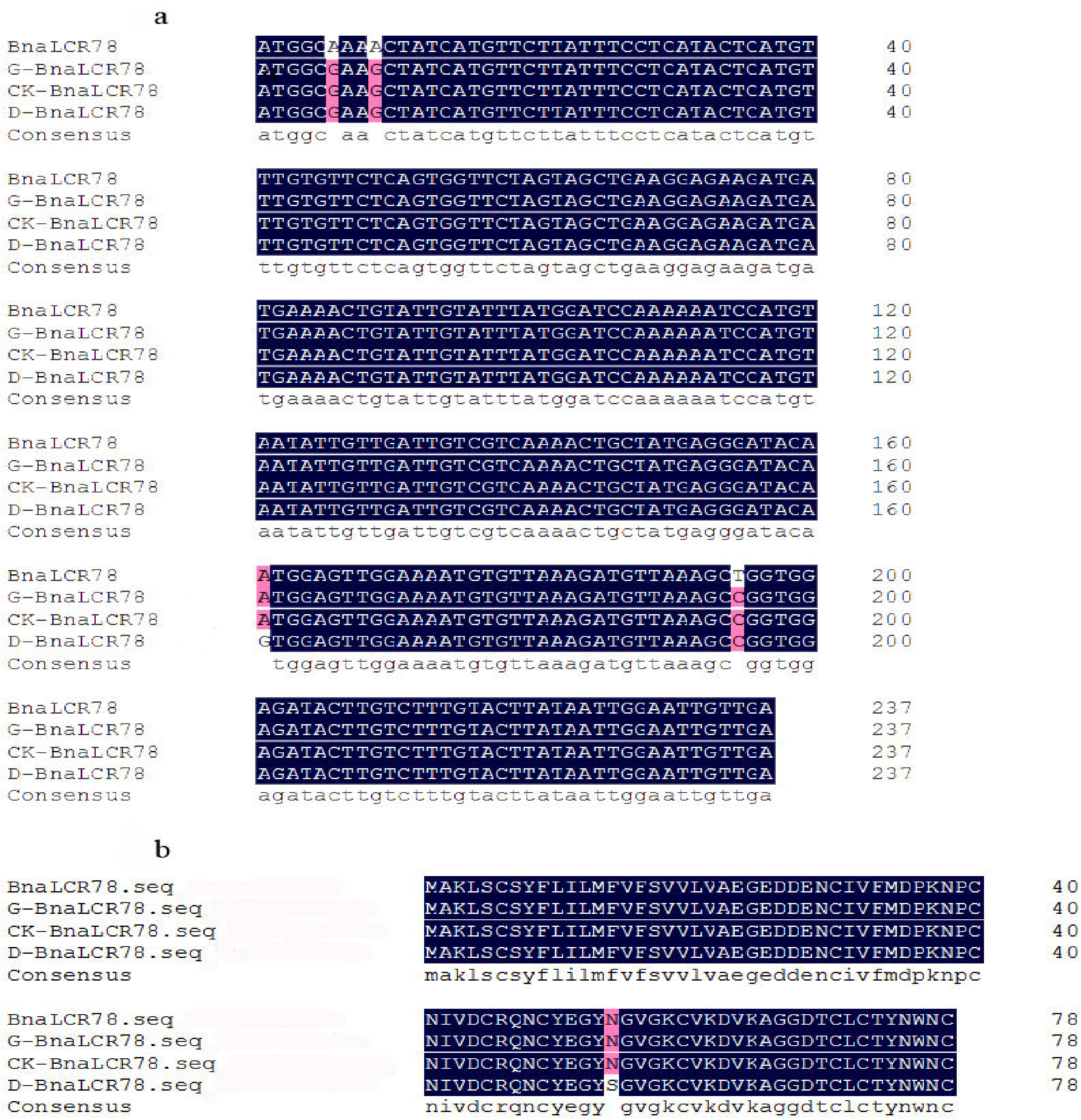

Fig. 1. Alignment of *BnaLCR78* in three types of rapes with *BnaLCR78* gene in electronic cloning.
a. Alignment in nucleotide level; b. Alignment in protein level
BnaLCR78: CDS sequence in electronic cloning; *G-BnaLCR78*、*CK-BnaLCR78*、*D-BnaLCR78* represents gene CDS sequences come from induced, high oleic acid content of rape, the original control and low oleic acid content rape, respectively

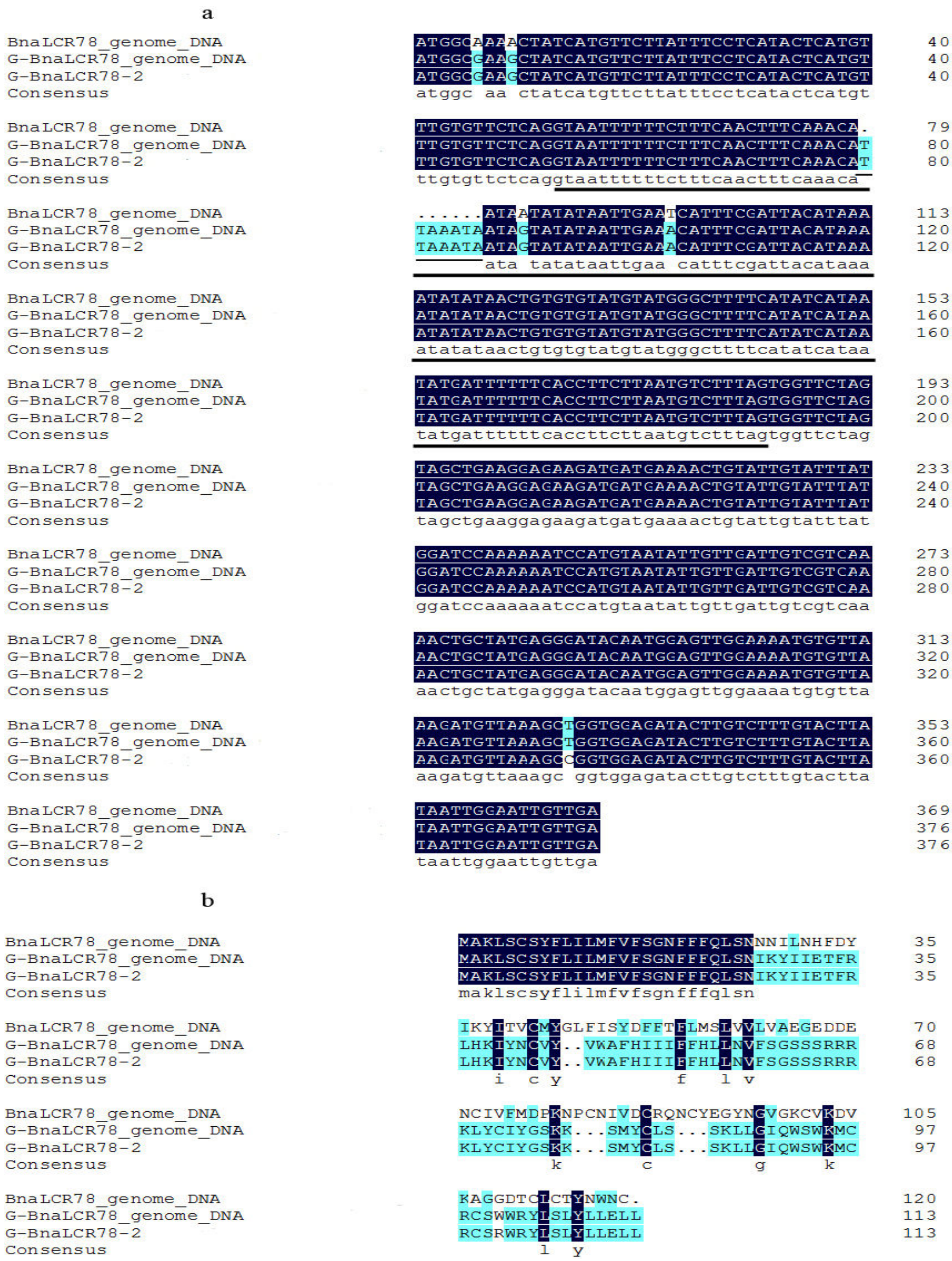

Fig. 2. Alignment of *BnaLCR78* in induced, high oleic acid content types of rape, the form of *BnaLCR78* retained intron with *BnaLCR78* gene in electronic cloning.

a. Alignment in nucleotide level; b. Alignment in protein level

BnaLCR78 genome DNA: *BnaLCR78* genome DNA sequence in electronic cloning; G- *BnaLCR78* genome DNA: *BnaLCR78* genome DNA sequence in induced, high oleic acid content types of rape; G- *BnaLCR78*-2: the form of *BnaLCR78* retained intron, respectively

Note: a wide black line and narrow black line represent retained intron and increased nucleotide, respectively

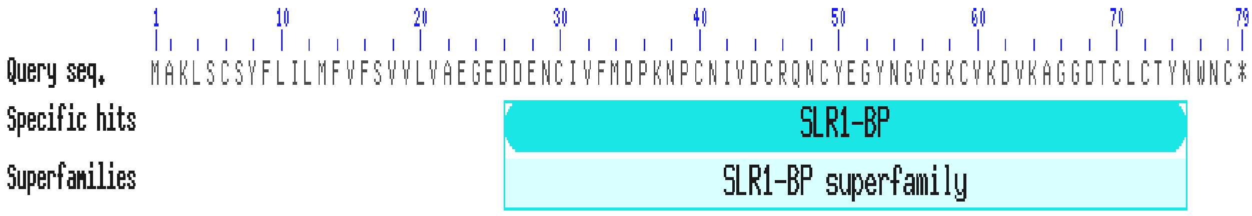

Fig. 3. The prediction result of conserved domain of BnaLCR78.

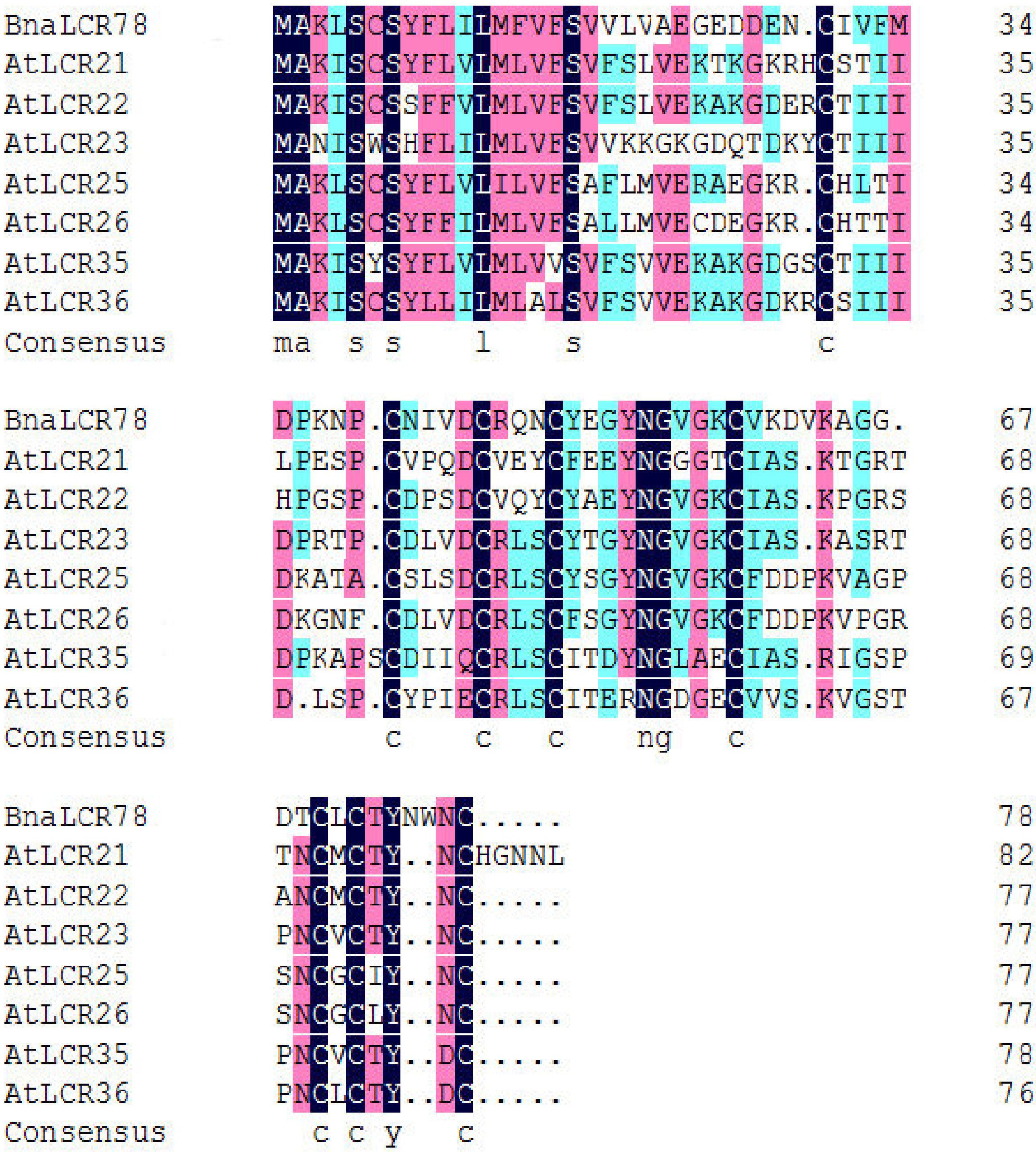

Fig. 4. Alignment of BnaLCR78 with that of some members of LCR family in Arabidopsis.
Brassica napus: BnaLCR78; *Arabidopsis thaliana*: AtLCR21 (NP_001031746); *Arabidopsis thaliana*: AtLCR22 (NP_194657); *Arabidopsis thaliana*: AtLCR23 (NP_001031745); *Arabidopsis thaliana*: AtLCR25 (NP_001031747); *Arabidopsis thaliana*: AtLCR26 (NP_194658); *Arabidopsis thaliana*: AtLCR35 (NP_001031393); *Arabidopsis thaliana*: AtLCR36 (NM_001036527).

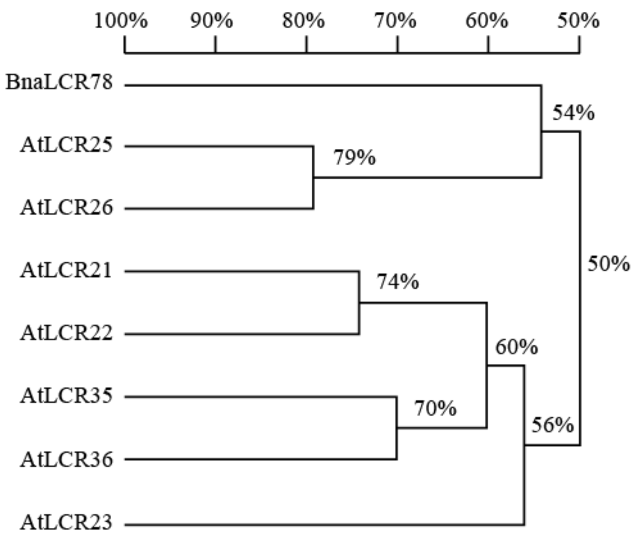

Fig. 5. Phylogenetic tree showing comparisions between predicted amino acid sequences from plant LCRs.

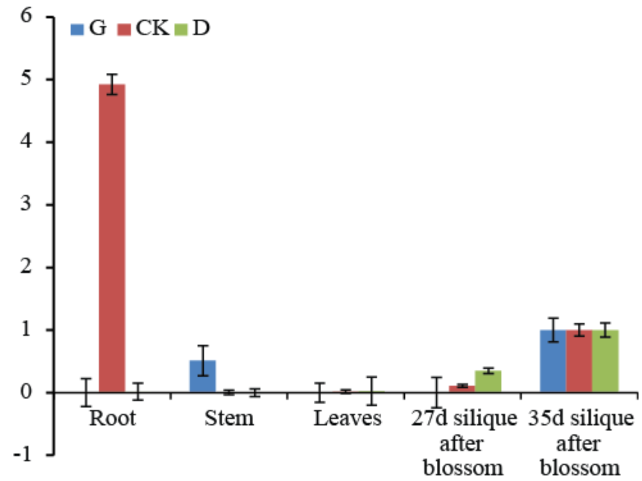

Fig. 6. Real-time RT-PCR profile of *BnaLCR78* in various tissues of three types of rapes (*β-actin* as a quantity control)
G, CK and D represent induced, high oleic acid content of rape, the original control and low oleic acid content of rape, respectively.

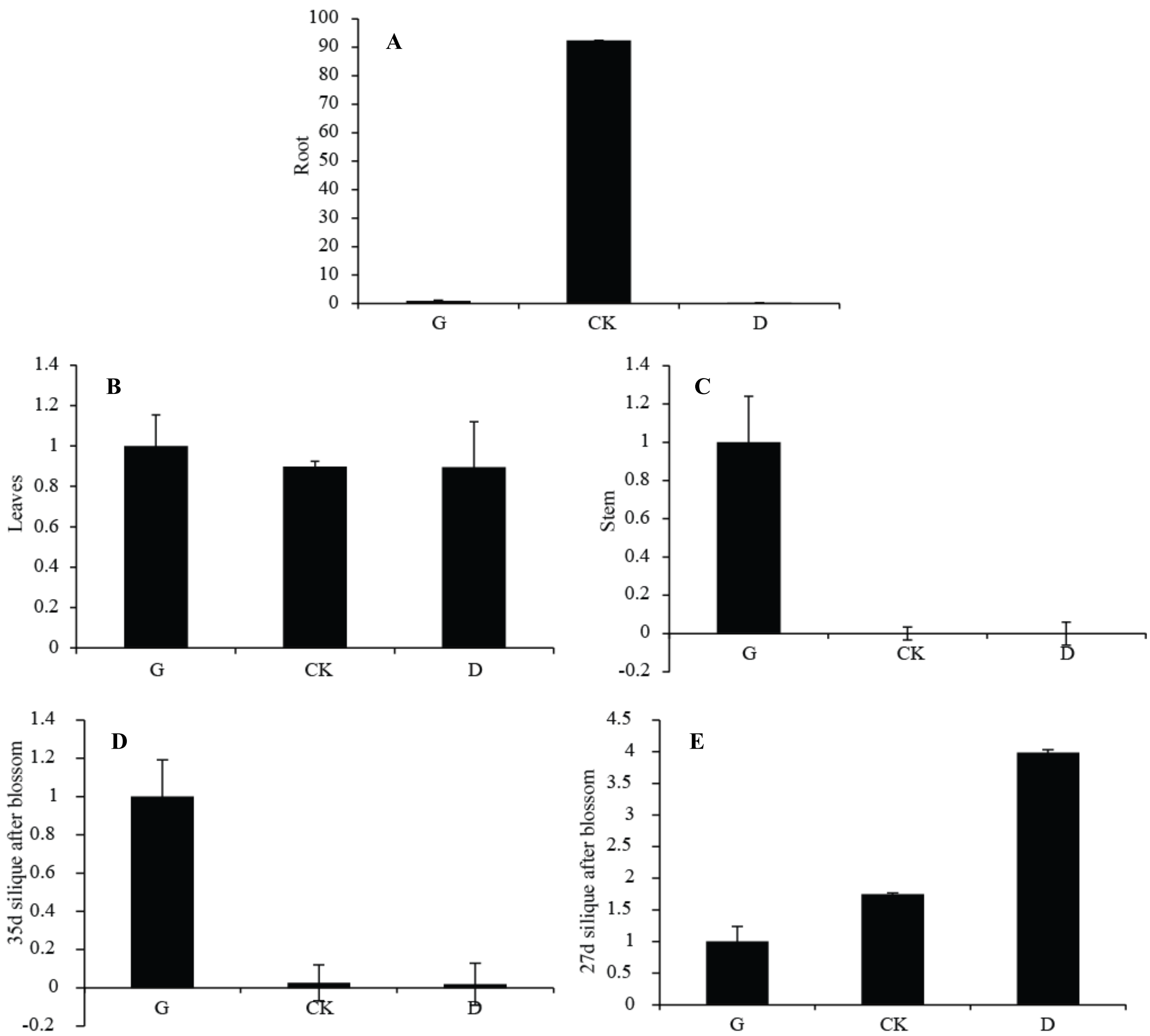

Fig. 7. Real-time RT-PCR profile of *BnaLCR78* in the same tissues of three types of rapes (*β-actin* as a quantity control).
G, CK and D represent induced, high oleic acid content of rape, the original control and low oleic acid content of rape, respectively.

By using bioinformatics tool, conserved encoding protein domain of *BnaLCR78* was analyzed, and results showed that BnaLCR78 protein had SLR1-BP conserved domain. SLR1-BP domain was the characteristic of SLR1-BP super-family, which indicated high homology between *BnaLCR78* and SLR1-BP super-family. Additionally, previous research showed that homology between LCR family in Arabidopsis and BnaLCR78 (Peng, 2011). Furthermore, members of the family had low homology in addition to contain eight highly conserved cysteines (Vanoosthuyse *et al*., 2001). Our work also confirmed these conclusions. The results of semi-quantitative RT-PCR and GUS histochemical staining indicated that expression of *AtLCR23* gene specific in pollinated flowers and young pods stigma, which suggested that the function of AtLCR23 may be related with pollen recognition (Yi *et al*., 2014). Many members of *LCR* including *AtLCR23* coded SLR1-BP domain, which specific recognition SLR1 of unlinked S loci in the phenomenon of SSI. SLR1 was the secreted protein existed in the stigma papilla cells. The pollen stigma adhesion greatly dropped when SLR1content decreased in stigma of antisense SLR1 transgenic plants (Takayama *et al*., 2009). Our work showed that homology between *BnaLCR78* and LCR family in Arabidopsis, which suggested *BnaLCR78* may play a role in pollination.

Previous semi-quantitative RT-PCR analysis with *Brassica napus* showed that *BnaLCR78* expressed only in silique rather than other tissues in plant, which suggested that it was expression specific in seed (Peng, 2011). For better understand the roles played by *BnaLCR78* gene in plants' development, further analysis of genes in three types of *Brassica napus*, induced, high oleic acid content, the original control and low oleic acid content of rape by quantitative RT-PCR method were conducted, and the results revealed that the highest expression of *BnaLCR78* was in 35 d silique after blossom was in G type. In stem, the gene expression was lower than that in 35 d silique after blossom. In CK type, the highest expression of *BnaLCR78* was in root. In 35 d silique after blossom, the gene expression was lower than that in root. In D type, expression of gene was the highest in 35 d silique after blossom. In 27 d silique after blossom, expression of *BnaLCR78* was lower than that in 35 d silique after blossom. Both in G type and D type, expression of gene in ground was higher obviously than that in underground. In CK type, expression of gene in contrasted to that in G type and D type. In three types of rape, the expression of gene was in other tissues including silique, which suggested the gene was unspecific in seed. Expression of *BnaLCR78* in three types of rape under different tissues indicated that the gene had different expression pattern in different rape type. Many genes had different expression pattern on time and space. The results were different from former research may be the reason of differences in sampling time.

By quantitative RT-PCR method, expression of *BnaLCR78* gene in three types of rape were analyzed, and results indicated expression of gene almost the lowest in leaves tissue and nearly equal in 35 d silique after blossom. For better distinguish expression of the gene, further analysis of the gene in specific same tissue by quantitative RT-PCR method were conducted, and the results showed that the trend on independent expression of *BnaLCR78* gene in root, in stem and in 27 d silique after blossom conformed to the expression of gene in three types of rape. And the differences among expression of gene in three types of rape were represented in leaves and in 35 d silique after blossom. In leaves tissue, the differences of expression of gene in three types of rape had no obvious distinguish though the highest expression level was in the G type. In 35 d silique after blossom tissue, the expression level of gene in G type was higher obviously than that in CK type and in D type, which had no obvious difference in the latter two. In leaves, in stem and in silique, expressions of *BnaLCR78* were detected. Water was released mainly into environment through stomata on leaves in plants. Moreover, vascular system of plant leaf also had an important role on the growth and development (Li *et al*., 2010). In stem, water was transported from root to leaf by catheter. Some elements such as N and P will be transported to stem, root and reproductive organ before leaf shedding (Li, 2011). Seed in silique was the organ that produced oil. Usually, seed oil and dry weight reached the maximum value in 30-45 days after blossom (Guan, 2006). The results of expression of *BnaLCR78* were detected in stem tissue and in leaves tissue suggested that the gene may involved in transpiration and transportation and distribution of nutrient, etc. All of the expression of genes in three types of rape higher in 35 d silique after blossom than that in 27 d silique after blossom, which suggested that *BnaLCR78* may also play an important role in oil content in rapeseed. By studying the relations between intron sequences and its corresponding coding sequences, two kinds of sequences segments are existed co-evolution relation (Zhao *et al*., 2010). Based on the ribosomal protein genes of 27 genomes, Smith-Waterman local alignment method were used to obtain the optimal matching segments between exon-exon sequences and their corresponding intron sequences. The intermediate section of intron sequences may play very important roles in gene regulation and gene expression (Zhang *et al*., 2013). And the study on *AtDWF4* also supports the conclusion (Peng *et al*., 2012). Like these genes mentioned above, whether the form of retained intron of *BnaLCR78* involved in gene expression and gene regulation need to further study.

Acknowledgment

This work was supported by Si Chuan Province Science and Technology Support Program (2012SZ0173). The rape seeds of three types in the study were provided by rape research center in Sichuan Agricultural University.

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(Received for publication 24 March 2015)