Molecular cloning and characterization of a novel gene involved in fatty acid synthesis in *Brassica napus* L.

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Abstract

Based on the sequence of a novel expressed sequence tag (EST), the full-length cDNA of 1 017 nucleotides was cloned from *B. napus* cv. Xiangyou 15 through rapid amplification of cDNA ends (RACE). The gene was designated as *Bnhol34* (HQ585980), encoding a protein of 338 amino acids. BLAST analysis showed no high degree of sequence identity to any known gene. The calculated molecular weight of the *Bnhol34* protein was 36.23 kDa, and the theoretical isoelectric point was 8.74. *Bnhol34* was also cloned from a high oleic acid mutant 854-1 through homologous cloning. There was no difference between the two *Bnhol34* genes. *Bnhol34* was localized in a tissue-specific manner in *B. napus*, and its expression level was about eight-fold greater in Xiangyou 15 seeds than in 854-1. The promoter region sequences of *Bnhol34* were then isolated from Xiangyou 15 and 854-1, and a 93-bp deletion was found to occur in the *Bnhol34* promoter region of 854-1. Three abscisic acid-responsive cis-elements (ABRE) were identified in the promoter region of Xiangyou15. Real-time PCR analyses revealed that exogenous abscisic acid increased *Bnhol34* expression by about four fold in Xiangyou 15 seeds, yet did not change *Bnhol34* expression in 854-1. It appeared that *Bnhol34* might be abscisic acid insensitive in 854-1.

Key words: *Brassica napus* L., rapid amplification of cDNA ends, high-efficient thermal asymmetric interlaced PCR, fatty acid synthesis, abscisic acid, expression

INTRODUCTION

*Brassica* species (primarily *B. napus*, *B. rapa*, and *B. juncea*) are now, after soybeans, the second largest oilseed crop in the world. After taking into consideration the safety of the edible vegetable oil supply, the Chinese government and agricultural companies have vigorously promoted rapeseed production in recent years, which reached a total seed yield for China of 1.37×10⁷ t in 2009 (Statistical Yearbook of China 2010). The typical rapeseed oil in China contains approximately 6% palmitic acid, 5% stearic acid, 61% oleic acid, 20% linoleic acid, and 8% linolenic acid. Linoleic and linolenic acids possess a very high nutritional value for human health; however, high levels of these polyunsaturated acids in oil make them susceptible to oxidation, and this causes a loss of flavor and rancidity to the oil, and results in a shortened shelf life (Browse *et al*. 1998). Oils with high oleic acid content, and low linoleic acid and linolenic acid content possess high nutritional value also, and can be heated to a higher temperature without smoking, thus making them more suitable for cooking. Therefore, breeding for rapeseed with high oleic acid,
and low linoleic acid and linolenic acid is one of main objectives for breeders (Burton et al. 2004; Scarth and Tang 2006). Our institute began to breed rapeseed with high oleic acid, and low linoleic and linolenic acids since 2000, and has obtained more than 100 B. napus lines with high oleic acid (>80%), and low linoleic acid and linolenic acid (total content <9%)

Plant oil synthesis that begins in the plastid is catalyzed by two enzyme systems: acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). The final products of ACCase and FAS are usually 16:0-ACP (acyl carrier protein) and 18:0-ACP; the products are then exported into the cytosol for desaturation and glycerolipid assembly (Ohlrogge 1997). The first desaturation step for fatty acids is catalyzed by a plastidial stearoyl-ACP desaturase, which introduces a double bond at carbon 9 and forms 18:1-ACP. The endoplasmic reticulum-bound oleate desaturase (FAD2) is the second desaturase in fatty acid desaturation, and this enzyme introduces the second double bond at carbon 12 and forms 18:2-ACP (Okuley et al. 1994; Mekhedov et al. 2000).

The composition of fatty acids produced in plants is primarily determined by thioesterases, condensing enzymes, and desaturases (Ohlrogge 1997). FAD2 is the primary enzyme that controls the relative content of oleic acid, linoleic acid and linolenic acid (Stoutjesdijk et al. 2002; Hu et al. 2006). However, regulation of fatty acid composition is a complicated biochemical process in which many still unknown genes are involved, and exploring these unknown genes is difficult yet exciting to researchers. We had isolated a novel gene from an oleic acid mutant SSH cDNA library in previous experiments (Xiao et al. 2009). In the present study, the full-length cDNA and promoter region sequences of the gene Bnhol34 were isolated, and the expression patterns of Bnhol34 in B. napus were investigated. The characterization of Bnhol34 might provide insight into the regulation of fatty acid composition in B. napus.

RESULTS

Isolation of the full-length cDNA of Bnhol34

Based on the 863-bp sequence isolated from a suppressive subtractive hybridization (SSH) cDNA library of B. napus, two primers were designed to obtain the 5’- and 3’- cDNA ends. A 405-bp fragment was isolated by using 3’ RACE and a 289-bp fragment was obtained by using 5’ RACE. By aligning and assembling the sequences of 3’ RACE, 5’ RACE and the core fragment, the full-length cDNA sequence of Bnhol34 was deduced and subsequently confirmed by sequencing; the full-length sequence was 1324 bp long. ORF analysis using the NCBI database showed that the Bnhol34 contained a 1017-bp ORF encoding a peptide of 338 amino acids, with a calculated molecular mass of 36.23 kDa and an isoelectric point of 8.74 (GenBank accession No. HQ585980). BLAST analysis showed no high degree of sequence identity to any known gene. The corresponding cDNA sequence in 854-1 plants was identified through homologous cloning. Alignment of the two Bnhol34 cDNA sequences through the DNAMAN program showed 100% sequence identity. Ahead of the ATG start codon and after the TAG stop codon, there were 125-bp 5’-UTR and 182-bp 3’-UTR sequences, respectively (Fig. 1).

Isolation of the Bnhol34 genomic clone and putative promoter regions

Bnhol34 genomic sequences were obtained from Xiangyou 15 and 854-1. The Bnhol34 DNA sequence when compared with its cDNA sequence showed that there was a 7-bp intron in the 5’-UTR of Bnhol34 (Fig. 2), and Xiangyou 15 and 854-1 Bnhol34 genomic sequences had 100% nucleotide sequence identity. The promoter is central to the regulation of gene expression, so for a better understanding of gene Bnhol34, a 1474-bp putative promoter region sequence was isolated first from Xiangyou 15 plants through high-efficient thermal asymmetric interlaced polymerase chain reaction (hiTAIL-PCR) (Fig. 3), and then the corresponding sequence of 1381 bp was isolated from 854-1 plants by homologous cloning. Compared with the two promoter sequences through the DNAMAN program, the results indicated a 93-bp deletion at positions-875 to -967 in the Bnhol34 promoter region of 854-1 (Fig. 2). The promoter region sequences were analyzed for known cis-acting elements through the web.
database program PlantCARE. Putative TATA box and CAAT box elements were determined at positions -263, -364, -628, -927, -1184, and -1367 upstream of the translation initiation site, respectively (Fig. 2). Based on web database analysis, some cis-elements in the Bnhol34 promoter were identified, including the Skn-1 motif (GTGACG, cis-acting regulatory element required for endosperm expression (Takaiwa et al. 1991), TCT motif (TCTTAC, light-responsive element (Kwon et al. 1994), MBS (CGGTCA motif, MYB-binding site (Nash et al. 1990), and ABRE (GACAACGTGTCC motif, TACGTA motif, ABA-responsive cis-elements, (Shen et al. 1993; Yamaguchi-Shinozaki et al. 1993), TGA-element (AACGAC, AAACAGA, auxin-responsive element (Pastuglia et al. 1997), and G-Box (CACACAAGA motif, CACACATGGA motif (Sommer and Saedler 1986; An et al. 1993). In addition, the Bnhol34 promoter also contained a CCGTCC box (CCGTCC motif) that is a cis-acting regulatory element related to meristem-specific activation.

Fig. 1 The nucleotide sequence of Bnhol34 cDNA (HQ585980) and the deduced amino acid sequence in B. napus Xiangyou 15.

Fig. 2 Nucleotide sequences of the promoter region and 5´-untranslated regions (5´ UTR) of the Bnhol34 gene. A small black box represents the transcription initiation site identified by 5´-RACE and is designated as the +1 position. The numbering on the left refers to the nucleotide sequences. The putative TATA box occurs in positions -263, -364, -628 and -1367. The putative ABA-responsive cis-elements (ABRE) occur in positions -763, -974 and -984. The translation initiation site (ATG) is in position +126. A 7-bp intron occurs between positions -118 and -126. The big black boxes at positions -875 to -967 represent a 93-bp deletion region in the Bnhol34 promoter region of 854-1.
Subcellular localization of Bnhol34

To determine the subcellular localization of the Bnhol34 protein in plant cells, in-vivo targeting experiments were performed. Bnhol34 protein was tagged at the N-terminus with GFP and then transiently expressed in onion epidermal cells under the control of the empty plasmid pCAMBIA1303. As shown in Fig. 4, the fusion protein Bnhol34::GFP was distributed throughout onion epidermal cells, indicating that Bnhol34 was expressed in both nucleus and cytoplasm.

Characterization of Bnhol34 gene expression patterns in various tissues

To examine the expression of Bnhol34 in various tissues of Xiangyou 15 and 854-1 plants, relative quantitative real-time RT-PCR analyses were performed. The results indicated that Bnhol34 was expressed in flower, silique coat and seed, but no expression was detected in leaf or root, suggesting tissue-specific expression. Furthermore, the expression of Bnhol34 was about 5-to -8 times higher in Xiangyou 15 silique coat and seed than in 854-1. (There is no Fig. 5 in the text)

Exogenous abscisic acid application increased Bnhol34 gene expression

Multiple ABA-responsive cis-elements were identified in the promoter region of Bnhol34. In order to assess the possible regulation of ABA on expression of the Bnhol34 during seed development, developing seeds (27 DAP) were incubated with various ABA concentrations. The real-time RT-PCR results revealed that exogenous ABA application resulted in a 3.2-to-4.5-fold (right?) increase in the expression of the Bnhol34 in the developing seeds of Xiangyou 15 plants, but such a change was not obvious in 854-1 seeds (Fig. 6). The expression pattern showed that the 93-bp deletion in the promoter sequence made the Bnhol34 gene ABA insensitive in 854-1 plants.

DISCUSSION

We isolated the full-length cDNA of the Bnhol34 gene and its promoter region sequences from B.napus
Xiangyou 15 and 854-1. The gene showed significant expression differences between Xiangyou 15 and 854-1 plants. Sequence analyses indicated that a 93-bp deletion was near three ABRE elements in the sequence of the 854-1 Bnhol34 promoter region, and that the deletion might make the ABRE elements nonfunctional.

The oleate desaturase gene FAD2 is the key factor responsible for the conversion of oleic acid to linoleic acid in non-photosynthetic tissues of plants (Okuley et al. 1994; Hu et al. 2006). Loss of FAD2 function results in an increase in oleic acid content in seed (Stoutjesdijk et al. 2002; Chen et al. 2006; Peng et al. 2010). When we investigated the expression levels of FAD2 in the developing seeds of Xiangyou 15 and 854-1, the results did not show any obvious differences between the two plants (data not shown); this suggested that FAD2 was not responsible for the change in the fatty acid composition in mutant 854-1 seeds. Xiangyou 15 and 854-1 plants have very similar agronomic traits, except for the fatty acid composition in their seeds. The oleic acid content of 854-1 seeds is about 38% higher than in Xiangyou 15, whereas the linoleic acid and linolenic acid content is about a quarter of that in Xiangyou 15. Bnhol34 manifested high expression levels in Xiangyou 15 seeds and low levels in 854-1, and was regulated by ABA in immature seeds; therefore, we hypothesized that Bnhol34 was a novel candidate gene involved in the regulation of fatty acid composition.

Bioinformatic analyses of the promoter sequence allowed the identification of conserved TATA and CCAT boxes as well as other transcriptional enhancer boxes. In addition to these conserved boxes, three ABRE elements were identified. Real-time PCR analyses revealed that exogenous ABA enhanced the expression of Bnhol34 gene in Xiangyou 15. 854-1 was derived from Xiangyou 15 ethylmethane sulphonate (EMS) mutant, and Bnhol34 was isolated from a high oleic acid mutant SSH cDNA library in our previous experiments (Xiao et al. 2009). RT-PCR indicated great different characterization of Bnhol34 between mutant 854-1 and wild-type Xiangyou 15. Analysis of Bnhol34 promoter showed that a 93-bp deletion was near three ABRE elements of 854-1 Bnhol34 promoter, and that the deletion might change the ABRE elements activity. ABA appears to play an important role in storage product deposition and maturation of developing seeds (Finkelstein and Somerville 1989). In Arabidopsis, the abi3 mutant is insensitive to ABA, and the mutation specifically affects seed development; additionally, seeds of Arabidopsis mutants have reduced amounts of storage proteins and lipids (Sugliani et al. 2010). Some researchers have reported that ABA promotes lipid synthesis and changes oleic acid and long-chain monounsaturated fatty acid synthesis in seeds (Kim and Janick 1991; Wang et al. 1996; Qi et al. 1998). The content of long-chain monounsaturated fatty acids (including eicosenoic acid and erucic acid) is very low in both Xiangyou 15 and 854-1 seeds. We expected the Bnhol34 gene is a member of ABA signal transduction pathway and involved in fatty acid synthesis. Bnhol34 changes the oleic acid content in 854-1 seeds, but not affects long-chain monounsaturated fatty acid synthesis in B. napus seeds.

In conclusion, the full cDNA and promoter region sequences of the novel gene Bnhol34 were isolated. The Bnhol34 was regulated by ABA, and considering its characterization in B. napus, we believe that the gene may be a regulatory gene involved in fatty acid metabolism.

**CONCLUSION**

In summary, the present study isolated a full cDNA and its promoter region sequences of a novel gene Bnhol34 through RACE and hiTAIL-PCR, and analyzed
the difference in \textit{Bnhol34} gene between high oleic acid mutant 854-1 and its original cultivar Xiangyou15. Bioinformatic analyses of the promoter sequence allowed the identification of three ABRE elements. In subsequent experiments, the real-time PCR experiments proved exogenous ABA regulating the expression level of \textit{Bnhol} in \textit{B.napus}. A 93-bp deletion occurred in the \textit{Bnhol} promoter of 854-1, which might make \textit{Bnhol} insensitive to ABA signal. The alter in promoter sequence and different expression profile of \textit{Bnhol} in high oleic acid mutant and its original cultivar make us believe that \textit{Bnhol} may be a regulatory gene involved in fatty acid metabolism.

**MATERIALS AND METHODS**

**Plant materials**

The line of \textit{B. napus} 854-1 described here was isolated after ethylmethane sulphonate (EMS) treatment of \textit{B. napus} Xiangyou 15 (Zhang et al. 2008). The mutant plants were self pollinated for five generations and controlled for higher oleic acid, lower linoleic acid and linolenic acid content in seeds. 854-1 and Xiangyou 15 have similar agronomic traits, except for the composition of fatty acid in their seeds. The seeds of 854-1 have a fatty acid composition of palmitic acid (C16:0) 2.95%, stearic acid (C18:0) 2.73%, oleic acid (C18:1) 84.77%, linoleic acid (C18:2) 4.96%, linolenic acid (C18:3) 2.75%, and eicosenoic acid (C20:1) 1.09%. The fatty acid composition of Xiangyou 15 seeds is: palmitic acid 4.2%, stearic acid 1.1%, oleic acid 61.1%, linoleic acid 21.1%, linolenic acid 8.4%, and eicosenoic acid 2.1%. 854-1 and Xiangyou 15 plants were grown simultaneously in the same greenhouse.

**Amplification of full-length \textit{Bnhol34} cDNA**

An oleic acid mutant SSH cDNA library had been constructed and screened prior to this experiment (Xiao et al. 2009), and an EST of 863 bp was isolated. Total RNAs were extracted from immature seeds at 27 d after pollination (DAP) of Xiangyou 15 plants using a Plant RNA Kit (OMEGA, USA) according to the manufacturer’s protocol, and the genomic DNA was digested from the total RNAs by RNase-free DNase (TaKaRa, Japan) at 37°C for 30 min. Total RNAs were used to synthesize 5’-RACE-Ready-cDNAs and 3’-RACE-Ready-cDNAs according to the manufacturer’s recommendation of the SMARTSTM RACE cDNA amplification kit (Clontech, USA). Based on the EST sequence already obtained, gene-specific primers GSP1, GSP2 and GSP3 were used to amplify the 5’-cDNA and 3´-cDNA ends, respectively, with the Advantage™ 2 PCR Enzyme kit (Clontech, USA). PCR was performed for 35 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 3 min, with an additional polymerization step at 72°C for 2 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, and purified using the DNA gel-extraction kit (OMEGA, USA). The purified products were cloned into the pMD18-T (TaKaRa, Japan) vector and then transformed into \textit{E. coli} DH5α. Recombinant plasmids were sequenced by Huada Gene Company (Beijing, China).

When the \textit{Bnhol34} cDNA sequence was obtained from the Xiangyou 15 plant transcriptome, the corresponding \textit{cDNA} sequence was isolated from 854-1 plants through homologous cloning by using primer pairs 854-1F and 854-1R (Table 1).

**Isolation of the \textit{Bnhol34} genomic clone and the \textit{Bnhol34} promoter sequence**

Total DNA was isolated from seven-day old rapeseed seed-

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**Table 1  List of primers used in experiments**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Sequence(5’→3’)</th>
<th>Description</th>
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</thead>
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<tr>
<td>GSP1</td>
<td>AGCTTAFGCCCTTATCCCATGTCC</td>
<td>Gene-specific primer for 5’ RACE</td>
</tr>
<tr>
<td>GSP2</td>
<td>CTTTGCAGAGCTTCTCATCTT</td>
<td>Gene-specific primer for 5’ RACE</td>
</tr>
<tr>
<td>GSP3</td>
<td>GAAGCTGCCGGGAAAATGGAG</td>
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<td>854-1F</td>
<td>AACCGAGGCTGCAAAAGTTT</td>
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<td>854-1R</td>
<td>GCCCTCAACAGTGATGCTC</td>
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<tr>
<td>854-1F2</td>
<td>TCCCTAGTCAGCAGCTCAAG</td>
<td>homologous cloning</td>
</tr>
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<td>holqF</td>
<td>GAGGAGGGCAATGTAAGGTTGAGA</td>
<td>Gene-specific primers for real-time PCR</td>
</tr>
<tr>
<td>holqR</td>
<td>CTCATGCTACTATGTAAGCTGCTGT</td>
<td>Gene-specific primers for real-time PCR</td>
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<tr>
<td>actinF</td>
<td>CGTGGCGATCTACGAGTTATG</td>
<td>à-actin gene primers for real-time PCR</td>
</tr>
<tr>
<td>actinR</td>
<td>CTCTAGCCGCTTACGCTCCT</td>
<td>à-actin gene primers for real-time PCR</td>
</tr>
<tr>
<td>SP1</td>
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<td>pre-amplification of TAIL-PCR</td>
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<td>SP2</td>
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<td>primary TAIL-PCR</td>
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<tr>
<td>SP3</td>
<td>TCTCTAACCAGCATATTGA</td>
<td>secondary TAIL-PCR</td>
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</table>
lings of Xiangyou 15. The Bnhol34 genomic sequence was amplified by PCR using 854-1F and 854-1R primers. The promoter region of the Bnhol34 gene was subsequently amplified from Xiangyou 15 plants through hiTAIL-PCR (high-efficient thermal asymmetric interlaced PCR). Sequence-specific primers (SP), such as 5'-nested PCR primers used for hiTAIL-PCR to amplify the Bnhol34 promoter, were located in the coding region of Bnhol34. The LAD degenerative primers used were the 3' primers referred to by Liu and Chen (2007). All the primers used for hiTAIL-PCR are shown in Table 1.

Three PCR procedures were included in the hiTAIL-PCR method for amplification of the Bnhol34 promoter. Pre-amplification reactions (20 µL) were prepared, each containing 2.0 µL PCR buffer, 200 µM each of dNTPs, 1.2 µM each of the LAD primers (in the case of LAD1 and LAD3, LAD2 and LAD4 were used in single reactions, and each was at a concentration 1.2 µM), 0.3 µM SP1, 1 U Taq, and 100 ng DNA. Each 25-µL primary TAIL-PCR contained 2.5 µL PCR buffer, 200 µM each of dNTPs, 0.3 µM AC1 and SP2, 1 U Taq, and 1 µL 40-fold diluted pre-amplified product. Each secondary 25-µL TAIL-PCRs contained 2.5 µL PCR buffer, 200 µM each of dNTPs, 0.3 µM AC1 and SP3, 1 U Taq, and 1 µL 10-fold diluted primary TAIL-PCR product. The PCR programs were followed as for Liu and Chen (2007) with minor modifications (Table 2). The PCR products were analyzed on 1.0% agarose gels, and single fragments were recovered from the gels and purified using a DNA gel-extraction kit (OMEGA, USA). The purified fragments were subcloned into pMD18-T vectors (TaKaRa, Japan), and were sent to Huada Gene Company (Beijing, China) for sequencing. Each clone was sequenced twice.

When the Bnhol34 genomic sequence and its promoter sequence were isolated from the Xiangyou 15 plant genome, the corresponding sequences cloned from 854-1 plants were identified through homologous cloning by using primer pairs 854-1F2 and 854-1R (Table 1).

### Bioinformatics analysis

The overlapping and assembly of cDNA fragments were performed using the "SeqMan" tool of DNAStar. Sequence identity was matched against the nucleotide and protein database of GenBank using BLAST tools. Conserved sequences were analyzed with the program of Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ExPASy ScanProsite (http://www.expasy.org/tools/scanprosite/). pl and MW were predicted using the DNAMAN program. Promoter prediction was performed on the SoftBerry TSSP (http://linux1.softberry.com/berry.phtml) and Berkeley Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) web servers; the known cis-acting elements were analyzed through a web search of publicly available databases (http://bioinformatics.psb.ugent.be/ webtools/plantcare/html).

### Subcellular localization of Bnhol34

The coding regions of Bnhol34 were amplified by PCR with primers: P1: 5'-'CCATGGATGATCTA TG ACAAAGC-'3' (NcoI); and P2: 5'-'ACTAGTATACCAAAGTCCATAAG-3' (SpeI). The termination codon of the Bnhol34 cDNA was removed after PCR. The PCR products were cloned into the vector pMD18-T (TaKaRa, Japan), digested with NcoI and SpeI, and then cloned into the expression vector pCAMBIA1303 (Clontech, USA) to produce expression vector pBnhol34-GFP. The plasmids were constructed to investigate the subcellular localization of Bnhol34 in onion (Allium cepa) epidermal cells. These plasmids were introduced into Agrobacterium tumefaciens strain LB4404. Transient expression assays were performed using the A. tumefaciens-mediated transformation method as described previously (Zhao et al. 2005). The onion epidermal

### Table 2 Thermal conditions for hiTAIL-PCR.

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<tr>
<th>Step</th>
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<th>Time (min:s)</th>
<th>Step</th>
<th>primary TAIL-PCR Temperature(°C)</th>
<th>Time (min:s)</th>
<th>Step</th>
<th>secondary TAIL-PCR Temperature(°C)</th>
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cells were visualized with a confocal laser scanning microscope (Leica, Germany).

Expression analysis for \textit{Bnhol}34

To investigate the expression of \textit{Bnhol}34 in various tissues and assess any possible hormonal control on expression of the \textit{Bnhol}34 during seed development, relative quantitative real-time RT-PCR was performed on ABI Prism 7300 (USA). Developing rapeseed siliques (27 DAP) were incubated in MS media supplemented with various concentrations of ABA (0, 20, 40, 60, 80 µM) for 24 h at 25°C. Siliques were placed flat on the medium and half of the silique was buried in the medium, and three independent treatments were performed. Total RNAs were extracted from roots, leaves, flowers, seeds and silique coats of 14-day-old rapeseed seedlings using a Plant RNA Kit (OMEGA, USA) following the manufacturer’s instructions. The seeds treated with ABA were dissected from siliques and then for RNA extraction. After residual genomic DNA was removed by treatment with DNase (TaKaRa, Japan), first-strand cDNAs were synthesized using ImProm-IITM Reverse Transcriptase (Promega USA) from 2 ìg of total RNAs. The cDNAs were assayed by relative quantitative real-time PCR using the 2×SYBR Green PCR Master Mix (Fermats, USA), 0.2 µM of each specific forward and reverse primer, and 4 ng of diluted cDNA for total RNA genes or 4 pg of diluted cDNA for the \textit{B. napus ACTIN} gene. PCR was carried out as follows: preheating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. The primer sequences are listed in Table 1. The relative expression was calculated using the 2-∆∆Ct method (Chao et al. 2009): 2-∆∆Ct = 2-((Ct/t-Ct/a)) . Ct: cycle threshold; Ct/t: cycle threshold of \textit{Bnhol}34; Ct/a: cycle threshold of \textit{ACTIN}.

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