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Full Length Research Paper

Cloning and analysis of plant fatty acid desaturase 7 gene promoter from *Brassica napus*

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In order to investigate the regulation mode of *Brassica napus FAD7* gene in response of thermal stress, we measured the protein levels of *BnFAD7* in plant at low and high temperature, and then analyzed promoter activity of 5'-flanking regions of *BnFAD7* by transient gene expression in *B. napus* protoplasts at different temperatures. Our studies indicated that no significant change occurred in the expression level of *BnFAD7* both at high and low temperature, while *BnFAD7* promoter showed a heat-induced regulation mode and slowly increased activity at the chilling conditions, which suggested there are heat-induced cis-action element lies in *BnFAD7* promoter sequence. Our data also suggested that a post-transcription regulation pattern existed to ensure BnFAD7 function in the acclimation to temperature stress. Furthermore, our studies give new evidence for the hypothesis that *BnFAD7* and *BnFAD8* gene may come from the same ancestor gene.

Key words: *Brassica napus*, fatty acids desaturase, promoter analysis, transient expression, *BnFAD7*.

INTRODUCTION

In many plants, environmental temperature shifts often affect the composition of fatty acids. On one hand, low temperatures bring a general increase in leve1 of polyunsaturated fatty acids observed in most plants during growth (Graham and Patterson, 1982). Fluidity of membranes has been considered to play an important role in survival at low temperatures (James et al., 1964; James and Craig, 1965). It is also reported that low temperatures could induced an increase in 18: 3 fatty acid that occur in glycerolipids of tobacco (Kodama et al., 1995) and in *britch* leaves (Martz et al., 2006). On the other hand, during acclimation to high temperature, some plants show a decrease in the proportion of trienoic fatty acids in their membrane glycerolipids (Pearcy, 1978; Raison et al., 1982; Iba, 2002).

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It is reported that the tendency of polyunsaturated fatty acids to decrease in abundance upon increasing growth temperature (37℃) is evident within both 16: 3 and 18: 3 (á-linolenic acid) fatty acids. The level of 18: 3 in *Arabidopsis* leaves decreased from 54 to 21 mol%, and 16: 3 species decreased from approximately 16 to 2.3 mol% at the same temperatures (Falcone et al., 2004). In *Brassica napus*, it is also observed that the portions of polyunsaturated fatty acids (mainly are 16: 3 and 18: 3) in total plant lipids decreased significantly with the evaluated temperature (form 5 to 30℃) (Mohabir and John, 1988).

Plant glycerolipids, including monogalactosyl diacylglycerol and digalactosyl diacylglycerol, contain over 75% trienoic fatty acids (Douce and Joyard, 1982). The membrane glycerolipids molecules contain a very high level of trienoic fatty acids, and more than 2/3 of the fatty acids present in the thylakoid membrane are 18: 3 or a combination of 18: 3 and 16: 3 (Roughanic acid) (Routaboul et al., 2012). The final step of 18: 3 and 16: 3 fatty acids biosynthesis is known mediated by three ù-3

fatty acid desaturase enzymes: FAD3, FAD7 and FAD8. The *FAD3* gene encodes an endoplasmic reticulum desaturase. The *FAD7* and *FAD8* genes both encode chloroplast desaturase isozymes that recognize either 18: 2 or 16: 2 as a substrate attached to any chloroplast lipid. Therefore, regulations on *FAD3*, *FAD7* and *FAD8* genes could directly affect the proportion of polyunsaturated fatty acids in plant.

It is reported that BnFAD3 protein abundance was regulated by a combination of cis-acting degradation signals in response to temperature and the ubiquitin-proteasome pathway (O'Quin et al., 2010). The mRNA level of *BnFAD8* (GenBank: FJ985692.1) was cold-induced at temperature lower than 16°C while undetectable at temperature above 20°C (Liu et al., 2011). So it is a meaningful question to find out whether the *BnAD7* gene could be induced by thermal-shifts. In total, investigation on *FAD7* gene regulation mode under temperature stress is helpful to understand the *FAD7* gene function in plant resistance for stress.

Many studies on the *FAD7* gene regulation under low or high temperature have been reported. However, different results have been obtained from a variety of plants, even in treatment of same temperature. It is reported that steady-state levels of the *AtFAD7* transcripts in *Arabidopsis thaliana* could not be induced at 12 and 30°C (Iba et al., 1993; Gibson et al., 1994). Same conclusion also was made by Nishiuchi on soybean *FAD7* (Nishiuchi et al., 1999) and by Monica on *PoleFAD7* in *Portulaca oleracea* (Teixeira et al., 2010), while *DsFAD7* transcript levels were observed down-regulated at 12°C in *Descurainia sophia* (Tang et al., 2007). In addition, the *LeFAD7* gene in *Lycopersicon esculentum* Mill showed an up-regulated mode in chilling conditions, and its mRNA levels decreased in response at elevated temperatures (Wang et al., 2010). Therefore, it is hard to anticipate the temperature-induced regulation mode of *B. napus FAD7* gene on the basis of known researches.

In this study, we measured protein levels of *BnFAD7* (GenBank: FJ985690.1) with western-blot analysis after treatments of different temperatures, then we isolated 5'-flanking regions of *BnFAD7* by genomic walking and analyzed promoter activity of the sequence. Promoter activities were tested by transient gene expression in *B. napus* protoplasts both upon low and high temperature. Our results suggested that a post-transcription regulation pattern might exist to ensure the function of *BnFAD7*.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *B. napus* (*B. napus*) line 84100-18 were presented by Prof. M. Wang. After 2 days jarovization at 4°C, seeds of *B. napus* were first surface sterilized in 75% ethanol for 1 min followed by immersion in 0.1 % $HgCl_2$ for 10 min, and rinsed at least three times with sterile distilled water, then the seeds were germinated in MS solid medium or in soils-mixture of vermiculite: peat (1: 1) in climatic chamber at 22°C with 16/8 h of light/dark cycle and 60% humidity.

Western-blot analysis

All *B. napus* plants were grow in 23°C for 1 month, and then part of them transferred to climatic chamber with temperature-set at 16 and 30°C, respectively for 48 h. Leaves at the same stage were collected and stored in -80°C for the material of total protein extraction. Total protein was isolated from the plants leaves at the same development stage. After the gel separation and blot transfer, the blot was incubated with primary rabbit source anti-BnFAD7 antibody and secondary antibody, peroxidase-conjugated anti-rabbit (Abcam; diluted 1:1,000). The target protein bands were visualized using the BCIP/NBP substrate solution (Roche). For target protein sample, the relative abundance of its expression is normalized by the protein level of internal control RbcS (Rubisco small subunit, from Seajet Scientific).

Isolation of 5'-flanking regions of *BnFAD7* by genomic walking

A genome walker kit from Clontech was used to isolate the proximal 5'-flanking regions of *BnFAD7* (GenBank: FJ985690.1), following the manufacturer's instructions. Briefly, five aliquots of *B.napus* genomic DNA were digested separately by four restriction enzymes (*EcoR*V, *Sma*I, *Dra*I and *Pvu*II) to produce five genomic DNA pools. Then, each pool which was ligated to an adapter sequence was used as a template in polymerase chain reaction (PCR) amplification of the 5'-flanking region of the *BnFAD7* by using the *BnFAD7*-specific primer (5' CGGGATCCTGTGTGCGCCAAAAGATCTG 3') and adapter primer 1(5'-GTAATACGACTCACTATAGGGC-3').

The polymerase chain reaction (PCR) product was diluted 50 folds and then used as the template for the second (nested) polymerase chain reaction (PCR) amplification using *BnFAD7*-specific primer 2 (5' CCCAAGCTTCGACGTGCAAGTTAACGGGT 3) and adapter primer 2 (5'-ACTATAGGGCACGCGTGGT -3'). The polymerase chain reaction (PCR)-amplified DNA fragment was cloned into vector pMD18-T and sequenced. Analysis of cis-elements was then operated on the cloned sequence by promoter scan software (Prestridge et al., 2000) and Plant care website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot et al., 2002).

Isolation of *B. napus* mesophyll protoplasts

The isolation of *B. napus* protoplasts was performed based on a modified protocol (Yoo et al., 2007). In brief, well-expanded leaves from 3 to 4 weeks old plants were cut into 0.5 to 1 mm strips taken from the middle part of the leaves. These strips were then incubated in an enzyme solution containing 250 mM MES (pH 5.7), 1% cellulase R10, 0.2% macerozyme R10, 0.4 M mannitol, and 20 mM KCl at 23°C for about 4 to 5 h with shaking (50 r/min).

Afterward, washing buffer I (167 mM mannitol and 133 mM $CaCl_2$) was added to the enzyme solution in an equal-volume and mixed gently. After filtered through a sieve with 150 µm pore diameter, the protoplast suspension was centrifuged at 60 g for 2 min, and the precipitant was then resuspended in washing buffer II (333 mM mannitol and 67 mM $CaCl_2$). Subsequently, the precipitant was washed twice with magma solution containing 5 mM MES (pH 5.7), 400 mM mannitol, and 15 mM $CaCl_2$. The viability of the protoplasts was verified with fluorescein diacetat staining. The final

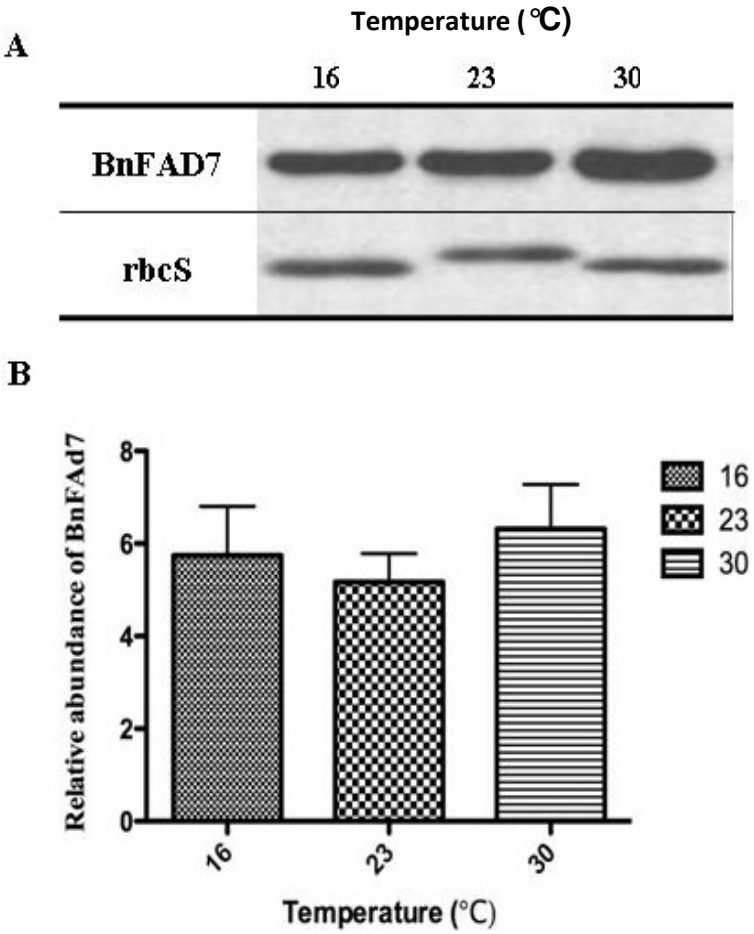

Figure 1. Assay of BnFAD7 expression level at different temperatures. Total protein was extracted from one month old *B. napus* leaves after 24 h thermal treatments and stored in -80℃. Results of western blot on *BnFAD7* expression level at 16, 23 and 30℃ are showed in A. Relative abundance of BnFAD7 was showed in B, relative abundance of BnFAD7 expression is normalized by the protein level of internal control RbcS (Rubisco small subunit). Vertical bars represent mean ± SD; data are from three independent experiments.

concentration of protoplast solution was adjusted to 10^6/ml using Magma solution.

Transient gene expression in protoplasts

The *BnFAD7* promoter activity was analyzed in *B. napus* protoplasts according to a modified protocol (Finkelstein et al., 2002). Briefly, for each transfection, 30 mg of *p*BI221-*pBnFAD7*: LUC DNA was added to 200 µl magma solution containing 5 x 10^5 protoplasts. While being shaken slowly by hand, Polyethylene glycol (PEG) solution containing 40% PEG4000, 0.2 M mannitol, and 0.1 M $CaCl_2$ in an equal volume was added to the transfection mixture, which was then incubated at room temperature for 10 min to ensure the uptake of the plasmid DNA.

After incubation, the transfected protoplasts were washed twice with 1 ml of WI solution containing 4 mM MES (pH 5.7) 500 mM mannitol, and 20 mM KCl, and resuspended in 0.25 ml of WI solution. Finally, the transfected protoplasts were transferred to 24-well tissue culture plates (0.25 ml in each well) and incubated in the dark at 23℃ overnight.

To measure *BnFAD7* promoter activity, five 5' deletion mutants of this genomic segment were amplified by polymerase chain reaction (PCR). Primers were designed in interval sequence between putative cis-elements regions (Figure 1) as follows:

BP1.5F: 5'- CCCAAGCTTACAGTGATAAGAGCGTTGAT -3'
BP1.2F: 5'- CCCAAGCTTGTGTGCGCCAAAAGATCTG -3'
BP0.9F: 5'- GATAGCGTGAATTGAACTTAGC-3'
BP0.6F: 5'- CCCAAGCTTCTGTAGACGCAACAATCATCAAAC-3'
BP0.3F: 5'- CCCAAGCTTTTGAGGTGGAAACCTTAGTGAAAT-3'
BPR: 5'- CGCGGATCC CGACGTGCAAGTTAACGGGT -3'

0.3, 0.6, 0.9, 1.2 and 1.5 kb DNA fragments were respectively amplified as shown in Figure 2A (each fragment kept a common 3'-end of promoter sequence by reverse primer of BPR). These

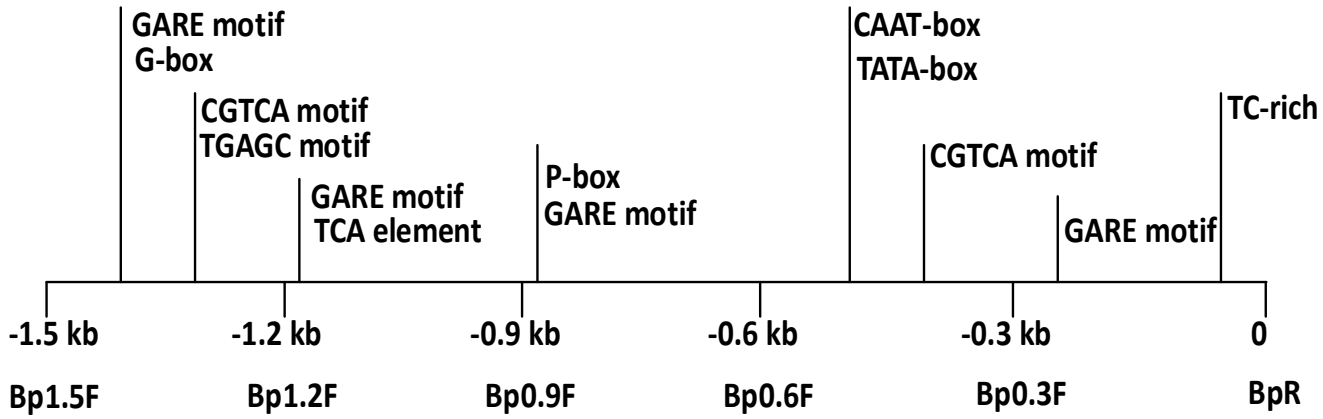

Figure 2. Location of regulatory elements in 5'-flanking region of *BnFAD7*. Horizontal line with coordinate indicated the upstream sequence of *BnFAD7*. All putative cis-elements on *BnFAD7* promoter sequence were based on the prediction of Plant care website. GARE-motif and P-box were gibberellins-responsive element, TC-rich repeats was cis-acting element involved in defense and stress response TCA-element was cis-acting element involved in salicylic acid response, G-box, TGAGC-motif, were both light responsive elements, and CGTCA-motif was MeJA responsive element. Sign lies under length coordinate (BP1.5F, BP1.2F, BP0.9F, BP0.6F, BP0.3F and BPR) indicated the primer locations.

fragments were subsequently cloned into vector pBI221 into *Hind*III/ *BamH*I site to promote the luciferase reporter gene (Figure 2A).

*p*BI221-empty or containing various lengths of 5'-flanking region of *BnFAD7* were transformed into *B. napus* protoplasts. The luciferase activity was measured by using Luciferase detection kit (Promega), after 12h incubation. The actual value was normalized by protein content of each sample and expressed as the relative luminescence units (RLU)/mg of protein. All results were repeated for three times and data were presented as mean ± SD.

Analysis of *BnFAD7* promoter under temperature stress

To understand whether the *BnFAD7* promoter contributes to temperature-responsive regulation or not, we placed luciferase gene under control of different length of *BnFAD7* promoter fragment (same *BnFAD7* promoter fragments used in Figure 1) and transfected these construct into protoplasts of *B. napus* respectively for transient analysis. Luciferase activity of each construct was measured timely on *B. napus* protoplast at low temperature (16°C), normal temperature (23°C), and high temperature (30°C), respectively. All results were repeated for at least three times and data were presented as mean ± SD.

RESULTS

Steady-state levels of the BnFAD7 protein were found at 16 and 30°C

Relative BnFAD7 protein level was evaluated by Western-blot and the results showed that no significant changes occurred in the BnFAD7 expression level at different temperatures (Figure 1). The hybridization bands of BnFAD7 and rbcS were exhibited in Figure 1A and the relative abundance of BnFAD7 protein was calculated with method of shadow integration showed in Figure 1B.

Isolation of the *BnFAD7*'s 5'-flanking region

Specific primers of *BnFAD7* were designed for the genomic walking experiment according to the cDNA sequence. Agarose gel electrophoresis proved the walking amplified DNA fragments. In addition, the product of nested polymerase chain reaction (PCR) by walking amplified is around 500 bp shorter than that of primary polymerase chain reaction (PCR), which agreed with the exception. Results of sequence analysis revealed the 3' end of nested polymerase chain reaction (PCR) overlapped the 5' end of *BnFAD7* cDNA by 233 bp. Assembly of the genomic walking and cDNA sequences generate a 5'-flanking sequence of 1451 bp (deposited to GenBank FJ965556).

In order to identify whether the DNA fragments amplified by genomic walking were upstream sequences, we analyzed the sequence overlap as described previously. Then we designed forward primers based on the sequence obtained by genomic walking and reverse primers within the cDNA region to polymerase chain reaction (PCR)-amplify genomic DNA extracted from leaves to verify these results. DNA fragments amplified by these polymerase chain reaction (PCR) reactions were sequenced and shown to contain the 5' end sequence of *BnFAD7* cDNA. These outcomes prove that the genomic segments isolated by genomic walking are truly the proximal 5' flanking regions of *BnFAD7*.

Promoter activity of the *BnFAD7* 5' flanking region

We tested the 5'-flanking region isolated by genomic walking to determine whether it contains the gene

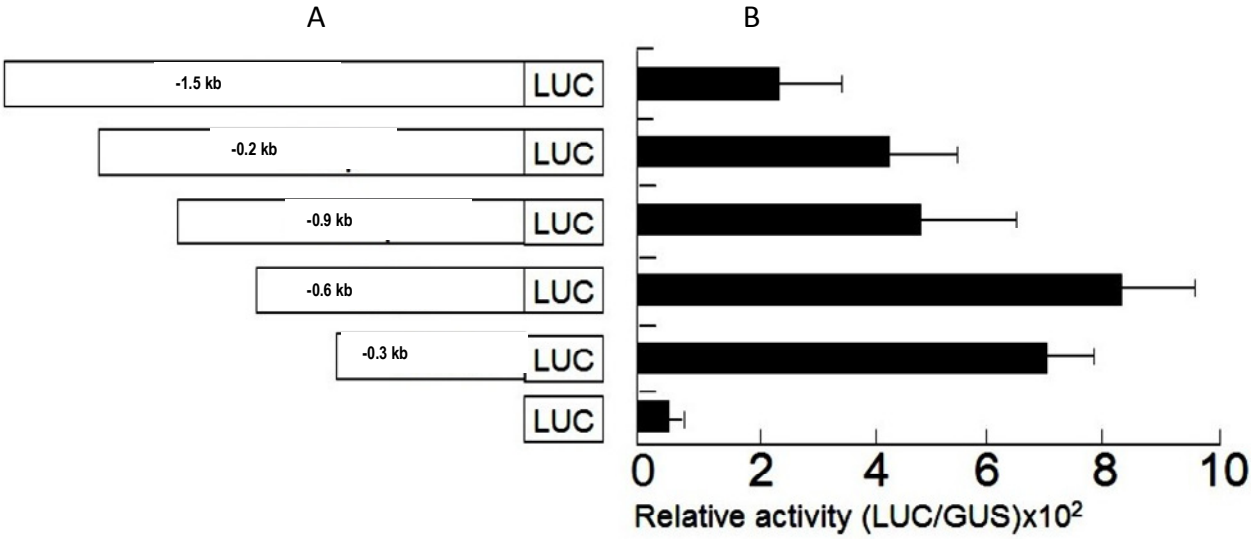

Figure 3. Core elements of *BnFAD7* promoter. (A) Schematic diagrams of truncated 5'-flanking regions of *BnFAD7*. Deletion constructs were generated by polymerase chain reaction (PCR), and cloned into the binary vector *p*BI221, driving the luciferase reporter gene. (B) Promoter activity analyzed by transient expression in *B. napus* protoplasts. *B. napus* protoplasts were transiently cotransfected with the indicated *BnFAD7* promoter constructs and expression vector for â-galactosidase. At 12 h posttransfection, Cells were harvested and the promoter activity was measured by luciferase assay, of which values were normalized to â-galactosidase values to adjust for the transfection efficiency. The data represented the mean value of three individual experiments (s.d.).

promoter. First of all, we generated a 5' deletion mutant of this genomic segment by polymerase chain reaction (PCR). Then we found that the amplified DNA fragments from these polymerase chain reaction (PCR)s of lengths 0.3, 0.6, 0.9, 1.2 and 1.5 kb were later cloned into the reporter gene vector *p*BI221 which contains the cDNA of Lucifer's (Figure 2). Empty vector and vectors containing various lengths of 5'-flanking region of *BnFAD7* were transfected into *B. napus* protoplasts as controls. 24 h later, we monitored the luciferase activity in these cells and the results are shown in Figure 3A.

Insertion of the 0.3 kb sequence upstream of the *BnFAD7* cDNA into *p*BI221 caused an about 10-fold growth in luciferase expression, indicating significant promoter activity in this region. The highest promoter activity which is 12-fold over the control was detected when the sequence reached 0.6 kb (Figure 3B). Since then, promoter activity gradually disappeared as the DNA length increased.

Activation of *BnFAD7* gene promoter by temperature stress

For the purpose of understanding *BnFAD7* functions in response to temperature stress, we placed a luciferase gene controlled by different length of *BnFAD7* promoter sequence, and transfected the construct into protoplasts of *B. napus* for transient analysis with the treatments of different temperatures (16, 23 and 30°C).

Results of luciferase activities shows that regulation mode of *BnFAD7* promoter is different between heat and low temperature conditions (Figure 4A). When plant cells incubated at low temperature, luciferase activities of each fragment slowly increase in first 4 h, and begin to drop after reaching the highest point at 5 h of chilling treatment. Moreover, our data showed that a more complex regulation pattern could be induced by high temperature. Luciferase activities of 0.6 kb fragment showed an up-regulated in first 4 h and then decreased after reaching the highest point at 5 h, at 7 h it increased again and there was still an increase trend of the luciferase activity after 8 h (Figure 4B).

DISCUSSION

Our studies suggested that heat-induced cis-action elements existed in *BnFAD7* promoter sequence. According to the results of Promoter scan software and Plant care website, many cis-acting elements were predicted in *BnFAD7* promoter sequence (Figure 2), but none of heat-responsive elements, like HSE element, was revealed in 1.5 kb upstream of *BnFAD7* CDS. Considering the CGTCA-motif and GARE-motif, both exists in 0.6 kb *BnFAD7* promoter, which could be induced by MeJA and gibberellins, respectively. To avoid these interferences, *B. napus* protoplast contains 0.6 kb,

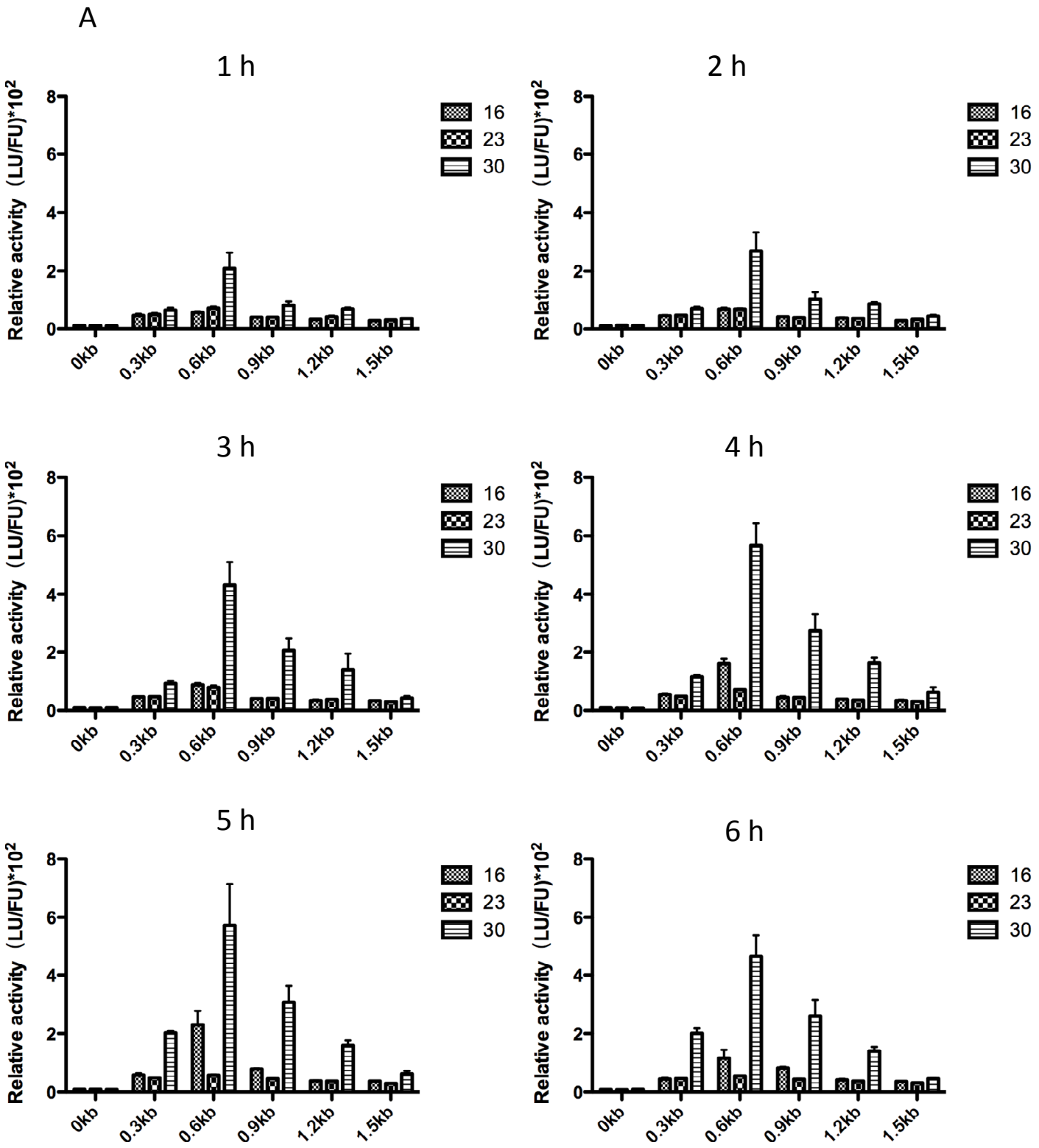

Figure 4. The inducible regulation of *BnFAD7* promoter activity in *B. napus* protoplasts. (A) Luc gene was controlled by different length of *BnFAD7* promoter fragments, Results of luciferase activities were assayed for each vector in every hour. Luciferase activities in normal temperature were also measured as a internal reference. (B) The protoplasts were incubated at the low temperature (empty triangle) or high temperature (filled triangle) for a continuous period. The data represented the mean value of three individual experiments (s.d.).

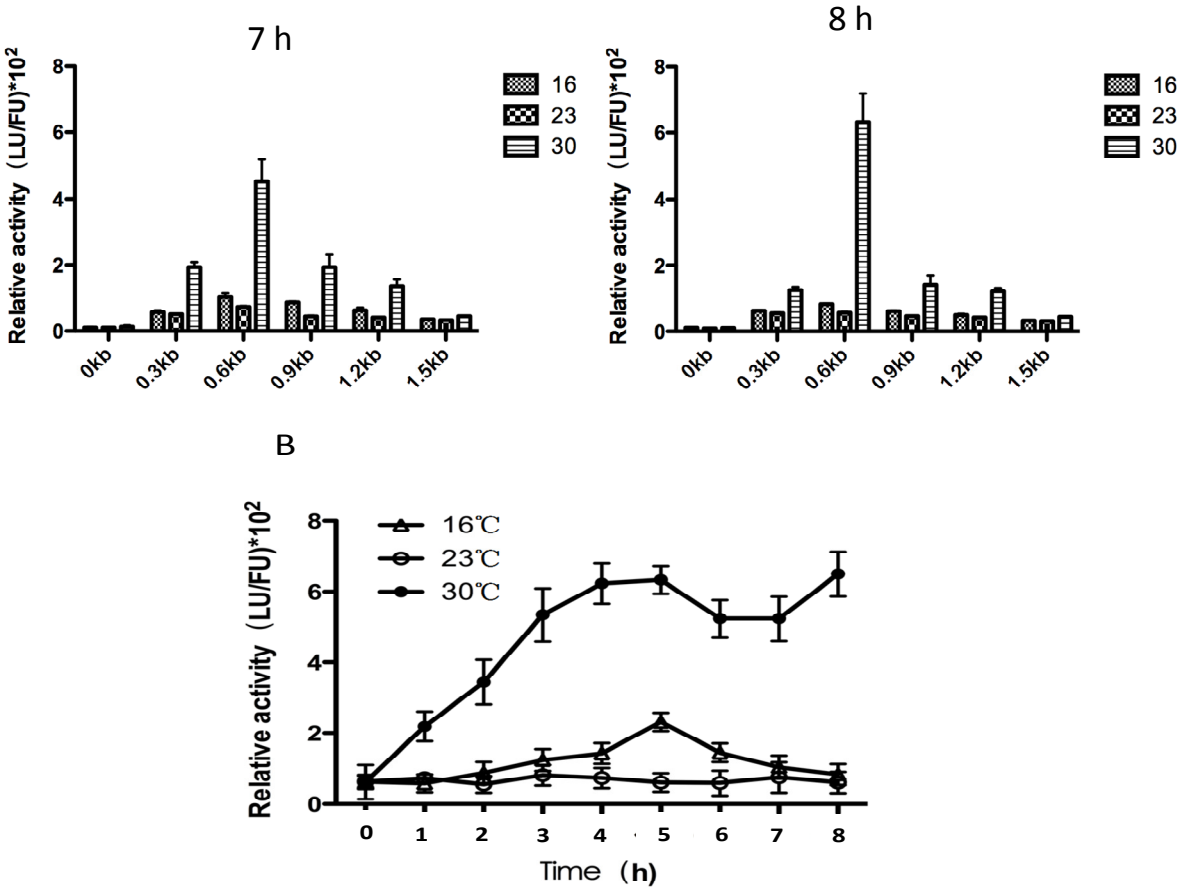

Figure 4. Contd.

BnFAD7 promoter was treated by Salicylic acid (SA) and Gibberellins (GA), respectively. The results indicated that only 10 μM SA could activated *FAD7* promoter, while GA treatment did not induce any evident regulation. Results of luciferase activities showed the highest points in 0.6 kb of *BnFAD7* promoter fragment, which suggested that some heat-induced cis-action elements lies in the 0.6 kb sequence upstream of *BnFAD7*.

It is reported that plant polyunsaturated lipids generally decreased upon high temperature (Mohabir and John, 1988). Our data showed that thermo-induced regulation also existed in *BnFAD7* promoter function but this rising trend, did not reflect in expression level of BnFAD7. Therefore, a post-transcription mechanism is suggested to reduce the accumulation level of BnFAD7 protein at high temperature.

In the temperature acclimation process of *B. napus* plant, *FAD8* gene plays an important role in chilling conditions with significant increased transcription level and protein accumulation (Gibson et al., 1994; Matsuda et al., 2005). According to the results of phylogenetic, tree assay on *B. napus* ω-3 desaturase genes, a close evolutionary distance lies between *BnFAD7* and *BnFAD8*, while a relative larger far distance lies between *BnFAD3* and *BnFAD7* or *BnFAD8*, indicating that they have different evolutionary sources.

The *BnFAD7* and *BnFAD8* gene may be derived from a common ancestral gene and many function variations have occurred in their evolutionary process (Berberich et al., 1998). The temperature-induced promoter activity of *BnFAD7* may be the retained function of the ancestor gene. To illustrate the process of evolution of the ancestral gene, more studies are needed to find out if these retained functions exist in homologous *FAD7* genes.

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