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Full Length Research Paper

Cloning and analysis of the ascorbate peroxidase gene promoter from *Brassica napus*

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Ascorbate peroxidase (*APX*) is known to catalyze the reduction of H_2O_2 to water and enhance plants' tolerance in stress environment. An ascorbate peroxidase protein (BnAPX) was previously isolated from *Brassica napus* in our laboratory and it was located in the chloroplast. In order to clarify the physiological function of *BnAPX* in plant response to photooxidative stress, 1562 bp upstream sequence of *BnAPX* was isolated by genomic walking and searched for cis-element by PROMOTER SCAN software and PLANTCARE website. Many light-responsive cis-elements were revealed in this prediction. Promoter activity analysis of this sequence was operated by transient expression in *B. napus* protoplasts. Results of promoter deletion analysis indicated that the core promoter element lied in 0.3 kb of *BnAPX* 5'-flanking region. Moreover, our data showed that promoter of *BnAPX* could be activated by light.

Key words: BnAPX, H_2O_2, promoter analysis, transient expression, genomic walking, 5'-flanking region.

INTRODUCTION

Plants are continually exposed to environmental stress, including high light, drought, salinity, heat and cold. These stresses disrupt the metabolic balance of cells, resulting in enhanced production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the superoxideradical (O_2^-), all of which could be diffused and accumulated apparently between different cellular compartments (Henzler and Steudle, 2000; Bienert et al., 2007). So, scavenging endogenous H_2O_2 timely can enhance plants' environmental tolerance to a certain degree.

Ascorbate peroxidase (APX, EC 1.11.1.11) catalyzes the reduction of H_2O_2 to water, using ascorbic acid (AsA) as the specific electron donor (Foyer and Hailiwell, 1976). APX has been identified in many higher plants and distributed in distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX) and cytosolic APX (cAPX) (Daveletova et al., 2005). In recent years, mAPX and cAPX as key regulators function in plant stress response has been clearly revealed. The expressions of apx1 gene of pea (Mittler and Zilinskas, 1992, 1994) and *Arabidopsis* (Storozhenko et al., 1998) and *APXa* gene of rice (Sato et al., 2001), which all contain heat-shock-elements (HSE) in their promoters, are induced by heat stress. The rice *APX* gene promoter contains a minimal heat shock factor-binding motif, 5´-nGAAnnTTCn-3´, the so-called heat shock element (HSE) (Sato et al., 2001). In *Arabidopsis*, a HSE found in the *APX1* promoter was shown to be recognized by the tomato heat shock factor (Hsf) *in vitro* and to be responsible for the *in vivo* heat-shock induction of the gene (Storozhenko et al., 1998)

However most of the researches focus on KO-*tAPX* and KO-*sAPX* using *Arabidopsis* knockout mutants and the roles of *tAPX* and *sAPX* remain unclear. It was found that the KO-*tAPX* and KO-*sAPX* single mutants and even the double mutants exhibited no visible symptoms of stress after long-term (1 to 14 days) high light (2,000 µmol photons m^{-2} s^{-1}) exposure. Moreover, lack of

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chlAPXs did not affect the levels and/or redox state of H_2O_2, AsA and glutathione (GSH), and the acti- vity of photosynthetic electron transport under the same high light conditions (Giacomelli et al., 2007). Although, the KO-*tAPX* and KO-*sAPX* single mutants exhibited phenotypes similar to those of wild-type plants under strong photooxidative stress (50 µM MV under high light), the double mutants showed severe phenotypes under the same conditions (Kangasjärvi et al., 2008). Unfortunately, in these studies, it remains unclear how the knockout mutants caused damage under photooxidative stress. Additionally, the contribution of each *chlAPX* to the tolerance to stress in higher plants is still under discussion.

A *Brassica napus* cDNA encoding an ascorbate peroxidase protein (BnAPX) was previously isolated in our laboratory using a yeast two-hybrid system and it was located in the chloroplast (Liu et al., 2010, 2011). In order to clarify the physiological function of *BnAPX* in plant response to photooxidative stress, we isolated the 5'-flanking regions of BnAPX by genomic walking and analyzed promoter activity of the sequence. Moreover, the promoter of BnAPX was light activated in protoplast systems.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *B. napus* line 84100-18 were presented by Prof. Maolin Wang. After 3 days vaporization 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. And then, the seeds were germinated in MS solid medium (Murashige and Skoog, 1962) or in soils-mixture of vermiculite : peat (1:1) in climatic chamber at 22°C with 16/8 h of light/dark cycle and 70% humidity.

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

A GenomeWalker kit from Clontech was used to isolate the proximal 5'-flanking regions of BnAPX, following the manufacturer's instructions. Briefly, five aliquots of *B. napus* genomic DNA were digested separately by five restriction enzymes (*EcoRV, HindIII, SmaI, DraI and PvuII*) to produce five genomic DNA pools. Then, each pool of DNA fragments was ligated to an adaptor sequence. The adapter-ligated genomic DNA was used as a template in PCR amplification of the 5'-flanking region of the BnAPX gene by using the BnAPX-specific primer (5'-CAGAGTGAGTGAGATGCG-GCGGAG-3') and adapter primer 1(5'-GTAATACGACTCACT-ATAGGGC-3'). The PCR product was diluted 50 fold and then used as the template for the second (nested) PCR amplification using BnAPX-specific primer 2 (5'-GAAGAAAAGGAAACGAGAG-AGGGAGAAG -3') and adapter primer 2 (5'-ACTATAGGG-CACGCGTGGT -3'). The PCR-amplified DNA fragment was cloned into pMD18-T and sequence.

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 at 23°C for about 4 to 5 h with shaking (50 r/min) in an enzyme solution containing 250 mM MES (pH 5.7), 1% cellulase R10, 0.2% macerozyme R10, 0.4 M mannitol and 20mM KCl. Afterward, buffer I containing 167 mM mannitol was washed and 133 mM $CaCl_2$ was added to the enzyme solution in an equal-volume and mixed gently. After filtering 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 containing 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 concentration of protoplast solution was adjusted to 10^6/ml using Magma solution.

Transient gene expression in protoplasts

The transient expression was analyzed in *B. napus* protoplasts according to a modified protocol (Finkelstein et al., 2002). Briefly, for each transfection, 30 mg of pBI221-pBnAPX: LUC DNA was added to 200 µl Magma solution containing 5 x 10^5 protoplasts. While being shaken slowly by hand, 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°C overnight.

To determine whether the 5'-flanking region isolated by genomic walking contains the gene promoter, various 5' deletion mutant of this genomic segment were amplified by PCR to measure the promoter activity. Considering the location of putative cis-elements in BnAPX promoter sequence, we amplified 0.3, 0.6, 0.9, 1.2 and 1.5kb DNA fragments (each fragment ends with translation initiation site of BnAPX) from the full length of BnAPX promoter, respectively. Each fragment was subsequently cloned into the reporter gene vector pBI221 in HindIII/BamHI site containing the cDNA of luciferase (Figure 1A) with the following primers: BP1.5F: 5'-CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BP1.2F: 5'-CCCAAGCTTTAGGCTTGTACTGACTATC -3'; BP0.9F: 5'-CCCAAGCTTATTCACAAATGGGTCACT -3'; BP0.6F: 5'-CCCAAGCTTAGAAAAGTTTTGACACTT -3'; BP0.3F: 5'-CCCAAGCTTACTGTACTCTTATTTCTAT -3'; BPR: 5'-CGCGGATCCGTTTACAACCAATAATT -3'.

PBI221-empty or containing various lengths of 5'-flanking region of BnAPX were transformed into *B. napus* protoplasts. Luciferase activity in these cells was monitored 24 h later. For light treatment, the transfected protoplasts were treated with 500 mmol photons m^{-2} s^{-1} for continuous times. The luciferase activities of the treated protoplasts were measured using Luciferase detection kit (Promega) according to the manufacturer's instructions. The actual value was normalized by protein content of each sample and expressed as the relative luminescence units (RLU)/mg of protein.

Analysis of *BnAPX* gene promoter by light

To understand whether *BnAPX* contribute to photoprotection or not, we placed a luciferase gene under control of the 0.3 kb BnAPX

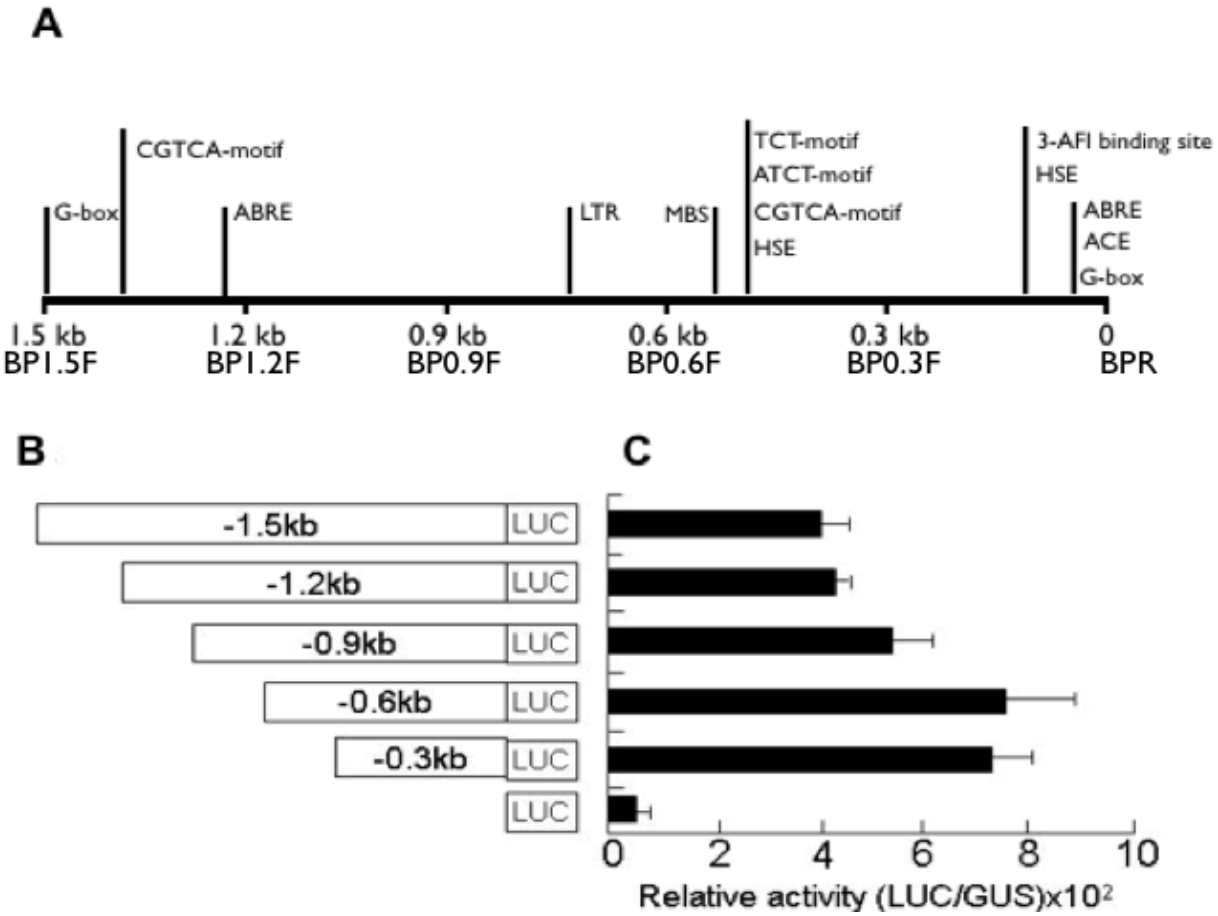

Figure 1. Promoter activity of the 5'-flanking region of BnAPX. (A) Location of regulatory elements in 5'-flanking region of BnAPX. Horizontal line with coordinate indicates the upstream sequence of BnAPX CDS. All putative cis-elements above BnAPX promoter sequence are from the forecast of PLANTCARE website. G-box, TCT-motif, ATCT-motif, 3-AF1 binding site and ACE are all light responsive element, HSE is a heat responsive element, LTR is a temperature responsive element, CGTCA-motif is a MeJA responsive element. Signs lying under length coordinate (BP1.5F, BP1.2F, BP0.9F, BP0.6F, BP0.3F and BPR) indicate the locations of primers. (B) Schematic diagrams of truncated 5'-flanking regions of Bn-APX. Deletion constructs were generated by PCR, and then cloned into the binary plasmid pBI221, immediately upstream of the promoterless luc gene. (C) Promoter activity analysed by transfection into *B. napus* protoplasts. *B. napus* protoplasts were transiently cotransfected with the indicated BnAPX promoter constructs and expression vector for β-galactosidase. At 24 h posttransfection, cells were then harvested and the promoter activity was measured by luciferase assay, of which values were normalized to β-galactosidase values to adjust to the transfection efficiency. The data represented the mean value of three individual experiments (s.d.).

promoter fragment (amplified by BP0.3F and BPR) and transfected the construct into protoplasts of *Arabidopsis thaliana* for transient analysis. The luciferase activity of protoplast was measured in different time with continuous light treatment (methods used in Arabidopsis protoplasts preparation and measurement were the same with *B. napus* protoplasts).

RESULTS

Isolation of the 5'-flanking region of BnAPX

Based on the cDNA sequence, gene-specific primers of BnAPX were designed for the genomic walking experiment. The walking amplified DNA fragments were verified by agarose gel electrophoresis. The product of primary PCR is about 300 bp longer than that of nested PCR by walking amplified (Figure 2), which was the expected result. Sequencing analysis revealed that the 3'end of nested PCR overlap the 5' end of BnAPX cDNA by 113 bp. Assembly of the genomic walking and cDNA sequences yielded a 5'-flanking sequence of 1562 bp (GenBank accession JN695783).

As described above, our initial approach to determine whether the DNA fragments amplified by genomic walking were upstream sequences was analyzed. To substantiate these results, we designed forward primers

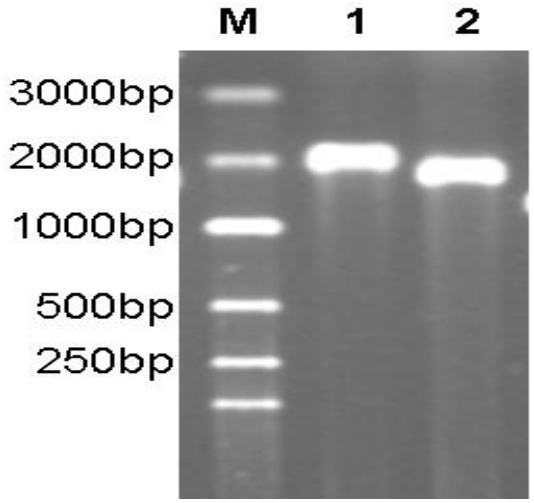

Figure 2. PCR products amplified from *B. napus* DNA by genomic walking. M, DNA molecular weight marker DL3000; lane 1, Primary amplification products; lane 2, nested amplification products.

based on the sequence obtained by genomic walking and reverse primers within the cDNA region to PCR-amplify genomic DNA extracted from leaves. DNA fragments amplified by these PCR reactions were sequenced and shown to contain the 5' end sequence of BnAPX cDNA. These results indicate that the genomic segments isolated by genomic walking are indeed the proximal 5'-flanking regions of BnAPX.

Promoter activity of the 5'-flanking region of BnAPX

In the analysis of BnAPX promoter activity, insertion of the 0.3 kb sequence upstream of the BnAPX cDNA into pBI221 led to about 10-fold increase in luciferase expression. When the sequence was extended to 0.6 kb, the highest promoter activity was detected, 12-fold over the control. Thereafter, with the increase in DNA length, promoter activity gradually diminished. Therefore, all results indicated that the core promoter element lied in 0.3 kb fragment of 5'- flanking region (Figure 1C).

Activation of *BnAPX* gene promoter by light

The results show that luciferase activity increased when treated with light in different times. The *B. napus* protoplasts was also used in this assay, the results of luciferase activity analysis in *B. napus* protoplasts was much lower than in Arabidopsis protoplasts though same light-inducible regulatory mode was exhibited (data not shown). The activity reached a maximum level after 6-h highlight treatment (Figure 3A). Correspondingly, the luciferase activity was at a very low level when treated with dark. Moreover, we confirmed that the light effect is specific to the BnAPX promoter and does not affect various other promoters (ubiquitin and CaMV35S) (Figure 4).

DISCUSSION

In this study, we obtained the 5'-flanking region of BnAPX by genomic walking, and vertified the promoter activity. Genomic walking is a PCR-based technique for rapid cloning of unknown genomic DNA from a known sequence (Siebert et al., 1995). It has been widely used to isolate promoters of genes for which only cDNA sequence is available (Bey et al., 1998; Zong et al., 1999).

Our result that the higher promoter activity for the 0.3 kb genomic segment was consistent with that from theoretical analysis using PROMOTER SCAN software (Prestridge et al., 2000). The database predicted the 0.3 kb segment to be a good promoter region. The sequence of this region is characterized by a high (57.8%) GC and possesses TATA and CAAT-box. Some putative cis-elements have been found in the 5'-flank region of the BnAPX gene by http://bioinformatics.psb.ugent.be/webtools/plantcare/html (Lescot et al., 2002), including ABRE, HSE, LTR and TC-rich, which indicated that BnAPX may participate in the response of stress (Figure 1A). However, the specifity and activity of BnAPX promoter need to be conformed in transient expression in plants cells by further experiments.

Our data show that the promoter of BnAPX was induced by light (Figures 3 and 4), which is consistent

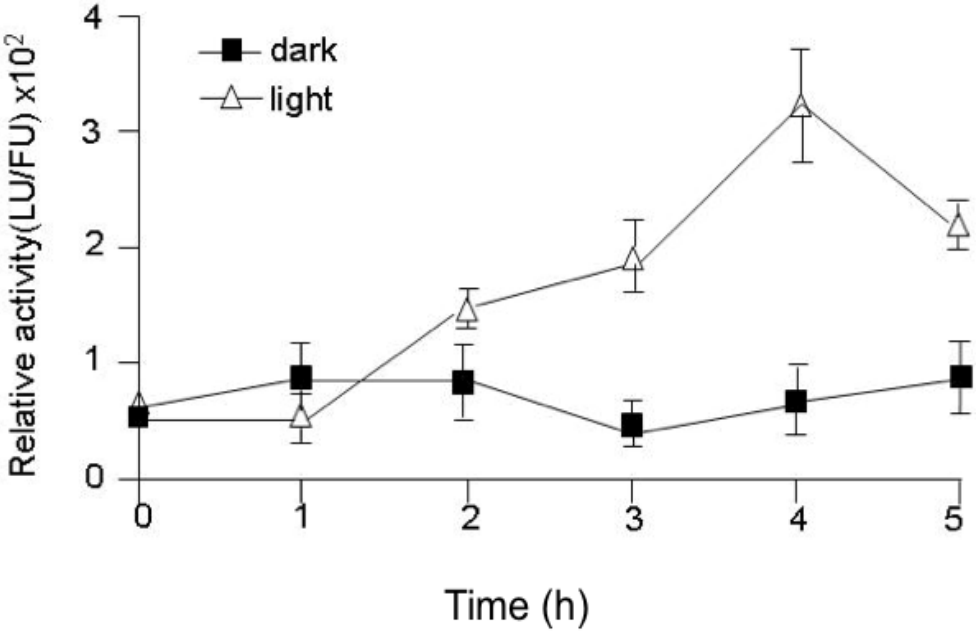

Figure 3. Regulation of BnAPX promoter activity by light in Arabiodopsis protoplasts. The protoplasts were treated with light (filled squares) or dark (empty triangles) for a continuous period.

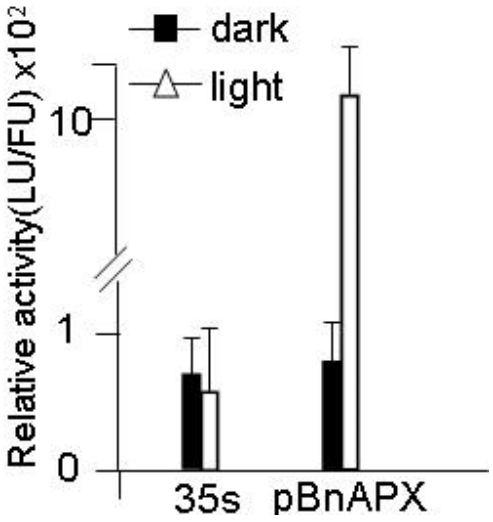

Figure 4. Specificity of light regulation relative to different promoter constructs in Arabiodopsis protoplasts. 35s, CaMV 35s-LUC; pBnAPX, promoter of BnAPX-LUC. LUC, luciferase.

with *in silico* analysis of the promoter of some elements involved in light responses, such as G-box, GAG-motif, Box4 and ACE (Sawant et al., 2005; Rushton et al., 2002), located in the upstream of the transcriptional start point. In the previous study, knockdown tAPX did not alter the phenotype under high-light stress, which implied that the tAPX expression was not up-regulated under light stress, but in the present research, the expression of BnAPX was increased. One possible explanation could be that several kinds of *tAPX* in the chloroplasts, have different functions under light stress, or that the induced BnAPX is inactive.

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