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 H₂O₂ 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, H₂O₂, 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 (H₂O₂), the superoxideradical (O₂⁻), all of which could be diffused and accumulated apparently between different cellular compartments (Henzler and Steudle, 2000; Bienert et al., 2007). So, scavenging endogenous H₂O₂ timely can enhance plants' environmental tolerance to a certain degree.

Ascorbate peroxidase (APX, EC 1.11.1.11) catalyzes the reduction of H₂O₂ to water, using ascorbic acid (AsA) as the specific electron donor (Foyer and Halliwell, 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
chiAPXs did not affect the levels and/or redox state of 
H$_2$O$_2$, 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 chi/APX 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 10 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 
soil 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'-CAGAGTGAGGAGATCGCC-
GCGGAG-3') and adapter primer 1(5'-GTAATACGACTCACT-
ATAGGAGC-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'-GAAGAAAAAGGAAACGAGAG-
AGGGAGAAG -3') and adapter primer 2 (5'-ACTATAGGG-
CAGCCGTGGT -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 20 mM 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 
Mgma 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 diacetate staining. The final concentration of protoplast 
solution was adjusted to 10$^{7}$/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 pbIB221-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 
temperature for 10 min to ensure the uptake of the plasmid 
DNA. After incubaion, the transfected protoplasts were washed 
twice with 1 ml of W1 solution containing 4 mM MES (pH 5.7) 500 
mM mannitol, and 20 mM KCl, and resuspended in 0.25 ml of W1 
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 deletions mutant of 
this genomic segment were amplified by PCR to measure the 
activity of promoter. 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 pbIB221 in HindIII/BamHI site containing the cDNA of 
luciferase (Figure 1A) with the following primers: BP1.5F: 5'-
CCCAAGCTTTCGAGAGAGTCT -3'; BP1.2F: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BP0.3F: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BP0.9F: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BP0.6F: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BP0.3F: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'; BPR: 5'-
CCCAAGCTTTGCGAGGAGATGCTTACT -3'); MG-
CCCGAGTACCCGCTTTTACACCAATATT -3'.

**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* 
expression as the relative luminescence units (RLU)/mg of protein.
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...
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 verified 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 specificity 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 Arabidopsis 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 Arabidopsis 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.
REFERENCES


