RESEARCH NOTE

Cloning and expression analysis of an anthocyanidin synthase gene homologue from *Brassica carinata*

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Introduction

Proanthocyanidins and anthocyanidins have an effect on the colour of flowers and seeds (Grotewold 2006; Lepiniec *et al.* 2006). Studying the biosynthetic mechanism of proanthocyanidins in *Brassica* is of great significance for elucidating the colour formation mechanism of seed coat and guiding the breeding work of yellow-seeded rapeseed (Marles and Gruber 2004; Yan *et al.* 2011). Anthocyanidin synthase (ANS), as an important enzyme in flavonoid biosynthesis, is a key point in studies on colour formation mechanisms of plant organs (Stracke *et al.* 2009). ANS is a ketoglutaric acid dependent dioxygenase, catalysing the transformation of leucoanthocyanidins to 3-OH-anthocyanidins, which is the precursor of anthocyanidins and proanthocyanidins (Abrahams *et al.* 2003). Functional defects or silencing of *ANS* affects plant colour formation, thus leading to colourless or white organs.

B. carinata is an important allotetraploidy of U triangle, and is suitable for planting in hot and dry areas. Thus, it is an important germplasm resource for genetic improvement of rapeseed in China. By distant hybridization, its superior genes can be transferred into the widely cultivated rapeseeds (Yao *et al.* 2012; Li *et al.* 2007). In the present study, *ANS* gene was cloned from the cDNA in the seed coat of black-seeded *B. carinata* by homology cloning. The characteristics, structure and function of its deduced protein were predicted by bioinformatics methods. Expression of *ANS* in leaves, embryo and seed coat was analysed, which provided a basis for further studies on the role of *ANS* in colour formation of *B. carinata* seed coat.

Materials and methods

Two *B. carinata* varieties, 3H008-6 (black-seeded line) and 3H007-2 (yellow-seeded line) were collected in Hunan Province, China. The RNA was extracted from leaves, seed coat and embryo of *B. carinata* according to the instruction of plant RNA extraction kit (Tiangen, Beijing, China). The DNA-free RNA samples were reversely transcribed into cDNA according to the instruction for transcriptase (ToYoBo, Shanghai, China).

The cDNA from seed coat of black-seeded *B. carinata* was used as template for gene cloning. Primer ANSU (sense primer: 5′-AAAGCTTCTTTACTTACTCT-3′, antisense primer: 5′-CTTTGTTACAAATCATACAA-3′) were designed according to the published sequence of *ANS* genes in NCBI. Polymerase chain reaction (PCR) system (20 μL total volume) included 2.0 μL of 10× PCR buffer (with $MgCl_2$), 0.3 μL of dNTP mix (10 mmol/L each), 1 μL of 10 mmol/L forward primer, 1 μL of 10 mmol/L reverse primer, 1 unit of *Taq* DNA polymerase and 1 μL cDNA, with sterilized deionized water being added to make a total volume of 20 μL. PCR procedure was as follows: 1 cycle of predenaturation at 94°C for 3 min; denaturation at 94°C for 35 s, annealing at 55°C for 45 s, and elongation at 72°C for 90 s, totally 36 cycles; followed by elongation at 72°C for 8 min. The PCR product recovered by gel extraction kit was linked to pMD18-T vector (TaKaRa, Dalian, China) and transformed into *E. coli* DH5α. Then, the recombinant plasmids were isolated and verified by PCR. The positive clones were sequenced and results were analysed by Dnaman software (Lynnon BioSoft, Vaudreuil, Quebec, Canada).

Molecular weight and isoelectric point (pI) were predicted online by Compute pI/Mw tool in Expasy (http://web.expasy.org/compute_pi/), and the conserved domains were analysed online (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). Secondary structures of ANS *B. carinata* (BcANS1) and ANS of *Arabidopsis thaliana* (AtANS) were predicted by logging onto http://swissmodel.expasy.org/workspace/index.php?func=tools_sequencescan1&userid=USERID&token=TOKEN using the model of PsiPred with the 2% *E* value. The transmembrane domain of BcANS1

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was predicted online in http://www.cbs.dtu.dk/services/TMHMM/. Structure of AtANS and BcANS1 signal peptides was analysed using neural networks (NN) model in http://www.cbs.dtu.dk/services/SignalP/.

Based on *BcANS1* sequences, primers ANS1D (sense primer: 5′- AAAGGCGGCTATGGATTGGG-3′, antisense primer: 5′- GGCTGAGGGCATTTCGGGTA-3′) were designed and the 459-bp fragment of *ANS* gene was amplified. For internal control, the primers ACT (sense primer: 5′-GACATTCAACCTCTTGTTTGCG-3′, antisense primer: 5′-CTGCTCGTAGTCAAGAGCAATG -3′) was used to simultaneously amplify the 666-bp fragment of *actin* gene. The *ANS* gene expression in leaves, 15 days after pollination (DAP) embryo and 15 DAP seed coat, 20 DAP seed coat and 25 DAP seed coat of 3H007-2 and 3H008-6 varieties was analysed by semiquantitive RT-PCR. PCR system (20 μL total volume) included 2.0 μL of 10× PCR buffer (with $MgCl_2$), 0.3 μL of dNTP mix (10 mmol/L each), 1 μL of 10 mmol/L forward primer, 1 μL of 10 mmol/L reverse primer, 1 unit of *Taq* DNA polymerase and 1 μL of cDNA, with sterilized deionized water being added to make a total volume of 20 μL. The PCR reaction was performed at 94°C for 3 min, and then subjected to 30 cycles of 94°C for 30 s, 61°C for 45 s and 72°C for 30 s, along with a final extension at 72°C for 6 min. The PCR procedure for *actin* was basically the same as that for *ANS*, except that the annealing temperature was 58°C.

ANS expression with shading treatment was evaluated by semiquantitive RT-PCR using *B. carinata* variety 3H008-6 (black seeded). Ten days and 20 days after pollination, the siliques of plants were shaded with tinfoil paper for five days. Control groups were grown under natural light without any shade. The RNA from seed coat and embryo after shading was reversely transcribed for expression analysis. The primers and PCR procedure for ANS and housekeeping gene *actin* amplification were the same as described in *ANS* gene expression analysis.

Results

In this study, a 1303-bp cDNA of *ANS* gene was obtained from *B. carinata*, named as *BcANS1*, it had an open reading frame of 1080 bp, 5′noncoding sequence of 48 bp, and 3′noncoding sequence of 175 bp (figure 1). By online Blast

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1       AAAGCTTCTTTACTTACTCTGTTTTAGCTTTAGAAGAAGTACAAGAAGATGGTTGCAGTT
                                                    M  V  A  V
61      GAAAGAGTTGAGAGCTTAGCAAAAAGCGGAATCGAATCAATCCCAAAAGAATACATTCGT
         E  R  V  E  S  L  A  K  S  G  I  E  S  I  P  K  E  Y  I  R
121     CCAAAAGAAGAGCTAGAGAGCATCAACGACGTGTTCCAAGAAGAGAAGAAAGAAGACGGT
         P  K  E  E  L  E  S  I  N  D  V  F  Q  E  E  K  K  E  D  G
181     CCACAAGTCCCCACCATCGATCTCCAAGACATCGAGTCAGAAGACGTAACTATCCGTGAG
         P  Q  V  P  T  I  D  L  Q  D  I  E  S  E  D  V  T  I  R  E
241     AAATGCATAGAGGAGCTCAAAAAGGCGGCTATGGATTGGGGAGTGATGCATCTGATCAAC
         K  C  I  E  E  L  K  K  A  A  M  D  W  G  V  M  H  L  I  N
301     CATGGTATACCTGTTGATCTAATGGAGCGTGTGAAGAAATCAGGAGAAGAGTTCTTCGGT
         H  G  I  P  V  D  L  M  E  R  V  K  K  S  G  E  E  F  F  G
361     TTGCCCGTTGAAGAGAAGGAGAAGTATGCAAACGATCAAGCCTCGGGAAAGATTCAAGGG
         L  P  V  E  E  K  E  K  Y  A  N  D  Q  A  S  G  K  I  Q  G
421     TATGGAAGCAAGTTAGCCAACAACGCGAGTGGACAACTTGAGTGGGAAGATTACTTCTTC
         Y  G  S  K  L  A  N  N  A  S  G  Q  L  E  W  E  D  Y  F  F
481     CATCTTGTGTATCCTGAAGACAAGAGGGATCTATCACTTTGGCCTAAGACACCAAGTGAT
         H  L  V  Y  P  E  D  K  R  D  L  S  L  W  P  K  T  P  S  D
541     TACTGTAGTGAAGCGACGAGTGAGTATGCCAAGTGTCTTCGTTTGCTAGCAACAAAAGTC
         Y  C  S  E  A  T  S  E  Y  A  K  C  L  R  L  L  A  T  K  V
601     TTCAAGGCTCTCTCTATCGGTCTAGGCTTAGAGCCTGACCGTCTAGAGAAAGAAGTTGGC
         F  K  A  L  S  I  G  L  G  L  E  P  D  R  L  E  K  E  V  G
661     GGCATAGAAGAGCTTCTCCTCCAAATGAAGATAAACTATTACCCGAAATGCCCTCAGCCT
         G  I  E  E  L  L  L  Q  M  K  I  N  Y  Y  P  K  C  P  Q  P
721     GAGCTAGCACTTGGCGTGGAAGCTCACACCGATGTAAGCGCTTTGACCTTCATTCTACAC
         E  L  A  L  G  V  E  A  H  T  D  V  S  A  L  T  F  I  L  H
781     AACATGGTACCTGGTTTGCAGCTGTTCTACGAGGGTAAATGGATCATTGCAAAATGTGTT
         N  M  V  P  G  L  Q  L  F  Y  E  G  K  W  I  I  A  K  C  V
841     CCTGATTCCATTGTGATGCACATTGGAGATACGTTGGAGATTCTTAGTAACGGGAAGTTC
         P  D  S  I  V  M  H  I  G  D  T  L  E  I  L  S  N  G  K  F
901     AAGAGTATACTTCACCGTGGGCTGGTGAACAAGGAGAAGGTTAGGATTTCTTGGGCTGTG
         K  S  I  L  H  R  G  L  V  N  K  E  K  V  R  I  S  W  A  V
961     TTCTGTGAGCCACCAAAGGATAAGATTGTGCTGAAGCCGTTGCCGGAGATGGTGAGTGTT
         F  C  E  P  P  K  D  K  I  V  L  K  P  L  P  E  M  V  S  V
1021    GAGTCTCCGGCTAAGTTTCCTCCAAGGACGTTTGCTCAGCATATTGCGCATAAGTTGTTT
         E  S  P  A  K  F  P  P  R  T  F  A  Q  H  I  A  H  K  L  F
1081    AAGAACGAGCAAGAGGAGTTGGTATCTGAGAAAAAGGATCAAGTTTGAGTCATTAAACAG
         K  N  E  Q  E  E  L  V  S  E  K  K  D  Q  V  *
1141    TATGTGAAAGTGTGAAACTCTTGTTCGAGTTTATCTGTTATCTTATGTTTGTGTATGTTT
1201    TCCTATCCTGTCTTTGATGTGTGTCTTATCAAAGATGTGAAGAGATATTTCTGTGGATTG
1261    TGTCATTGTGGTATCTTAAAGAATTGTATGATTTGTAACAAAG
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Figure 1. cDNA sequence and deduced amino acid sequence of *B. carinata BcANS1* gene. The bold underline letter triplets show start and stop codons.

with published *ANS* sequences in GenBank, it was found that *BcANS1* was the highest homologous (92% similarity) to *ANS* from *Brassica juncea* (EU927147).

BcANS1 encoded a peptide containing 359 amino acid residues, whose molecular weight and pI were predicted as 40766.92 Da and 5.05, respectively. Homology analysis of BcANS1 protein was carried out with Blast in NCBI (compositional score matrix adjustment). According to the results, BcANS1 showed 96% similarity in amino acid sequence with ANS from mustard (*Brassica juncea*, ACH58397), cabbage (*Brassica rapa* subsp. *oleifera*, ABY89681) and kale (*Brassica oleracea* var. *capitata*, AAO73440), 93% similarity with ANS from *Matthiola incana* and *A. thaliana*. Conserved domain analysis of BcANS1 showed the existence of 2-oxoglutarate and Fe (II)-dependent oxygenase domain in BcANS1, which also exist in ANS from other plants such as *A. thaliana*.

Secondary structure of BcANS1 and *A. thaliana* ANS protein (AtANS) were predicted online. Ten successive α-helices and 11 successive β-sheets were found in both proteins, indicating the high similarity in secondary structures between BcANS1 and AtANS. No transmembrane domain existed in BcANS1. Just as AtANS, BcANS1 might belong to cytoplasmic protein. According to the results of signal peptide analysis, neither BcANS1 nor AtANS had signal peptide, and thus both proteins belonged to nonsecretory protein.

The *ANS* gene expression in leaves, embryo and seed coat of 3H007-2 and 3H008-6 varieties were analysed, with cDNA as the templates, using ANS1D as primer and *actin* gene as internal reference. Results showed that *ANS* gene was expressed in leaves, embryo of yellow seeded and black seeded varieties, without significant difference between two varieties (figure 2). No *ANS* expression could be detected in seed coat 15, 20 and 25 days after pollination in yellow seeded variety 3H007-2, while the *ANS* expression was detected in seed coat in 15, 20 and 25 days after pollination in black seeded variety 3H008-6 (figure 2). This indicated that the blocking of *ANS* gene expression might be the reason for the lack of proanthocyanidins synthesis in 3H007-2 seed coat, which led to the increase in transparency of seed coat, and thus the seeds showed the colour of yellow embryo.

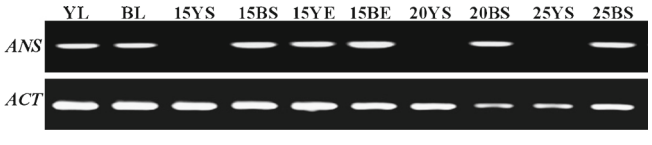

Figure 2. Transcription levels of *ANS* in leaves, embryos and seed coats of *B. carinata.* YL, leaf of 3H007-2; BL, leaf of 3H008-6; 15YS, 15 DAP seed coat of 3H007-2; 15BS, 15 DAP seed coat of 3H008-6; 15YE, 15 DAP embryo of 3H007-2; 15BE, 15 DAP embryo of 3H008-6; 20YS, 20 DAP seed coat of 3H007-2; 20BS, 20 DAP seed coat of 3H008-6; 25YS, 25 DAP seed coat of 3H007-2; 25BS, 25 DAP seed coat of 3H008-6.

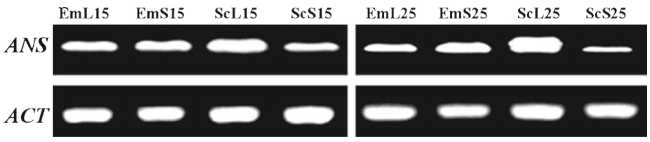

Figure 3. Expression pattern of *ANS* in embryos and seed coats of *B. carinata* under light treatment. EmL15, 15 DAP embryo under natural light; EmS15, 15 DAP embryo under shading treatment; ScL15, 15 DAP seed coat under natural light; ScS15, 15 DAP seed coat under shading treatment; EmL25, 25 DAP embryo under natural light; EmS25, 25 DAP embryo under shading treatment; ScL25, 25 DAP seed coat under natural light; ScS25, 25 DAP seed coat under shading treatment.

To investigate the effects of light on *BcANS1* expression, shading treatment was carried out using black seeded variety 3H008-6 as material to analyse the *BcANS1* expression. Results of semiquantitative PCR showed that *ANS* expression in 15 and 25 days after pollination differed between seed coat and embryo (figure 3). In embryo, *ANS* expression was not significantly different between shading and light treatments. In seed coat, *ANS* expression was significantly inhibited by shading treatment, and inhibition degree of 25 DAP seed coat was obviously higher than that of 15 DAP seed coat (figure 3). The results demonstrated that the *ANS* expression was induced by light.

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