Functional Identification and Characterization of the *Brassica Napus* Transcription Factor Gene BnAP2, the Ortholog of *Arabidopsis Thaliana* APETALA2

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**Abstract**

BnAP2, an APETALA2 (AP2)-like gene, has been isolated from *Brassica napus* cultivar Zhongshuang 9. The cDNA of BnAP2, with 1,299 bp in length, encoded a transcription factor comprising of 432 amino acid residues. Results from complementary experiment indicated that BnAP2 was completely capable of restoring the phenotype of *Arabidopsis ap2-11* mutant. Together with the sequence and expression data, the complementation data suggested that BnAP2 encodes the ortholog of AtAP2. To address the transcriptional activation of BnAP2, we performed transactivation assays in yeast. Fusion protein of BnAP2 with GAL4 DNA binding domain strongly activated transcription in yeast, and the transactivating activity of BnAP2 was localized to the N-terminal 100 amino acids. To further study the function of BnAP2 involved in the phenotype of *B. napus*, we used a transgenic approach that involved targeted RNA interference (RNAi) repression induced by ihp-RNA. Floral various phenotype defectives and reduced female fertility were observed in *B. napus* BnAP2-RNAi lines. Loss of the function of BnAP2 gene also resulted in delayed sepal abscission and senescence with the ethylene-independent pathway. In the strong BnAP2-RNAi lines, seeds showed defects in shape, structure and development and larger size. Strong BnAP2-RNAi and wild-type seeds initially did not display a significant difference in morphology at 10 DAF, but the development of BnAP2-RNAi seeds was slower than that of wild type at 20 DAF, and further at 30 DAF, wild-type seeds were essentially at their final size, whereas BnAP2-RNAi seeds stopped growing and developing and gradually withered.

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**Introduction**

Flower is the most important organ of a flowering plant. As a close relative of *Arabidopsis thaliana*, *Brassica napus* has equally a concentric arrangement of four types of flower organs: four sepals in whorl 1, four petals in whorl 2, six stamens in whorl 3, and two fused carpels in whorl 4. Floral organ identity is specified by the transcription factors encoded by 3 classes of floral homeotic genes: the A, B and C functional genes [1–3]. Class A genes specify sepals and also interact with class B genes to specify petals. Class C genes specify carpels and also interact with class B genes to specify stamens. Classes A and C genes act antagonistically to restrict each other’s activities in perianth and reproductive organs respectively.

APETALA2 (AP2) gene is one of the primary members of class A genes in *Arabidopsis*, characterized by the AP2 DNA binding domain of transcription factors unique to plants, it specifies whorl 1 and 2 organ identity in *A. thaliana* [4,5]. During floral development, AP2 is essential for the determination of the identity of sepals and petals. In the weak ap2 mutants, leaf-like organs replace sepals, and petals exhibit androecoid characteristics, and in the strong ap2 mutants, carpels are formed in the outer whorl of the flower, petals are absent, and the number of stamens is reduced [6–9]. It is especially valuable for oilseed rape (*B. napus*) breeding that AP2 is also involved in ovule and seed development [5,10], regulation of seed size [11,12], and the maintenance of the stem cell niche of the shoot meristem [13]. It has been shown that AP2 and its closest homologs are the targets of miR172, which down regulates these target genes by a translational inhibition mechanism rather than by RNA cleavage [14].

AP2 homologs that share similarities in gene structure and function with AP2 have been isolated from numerous species. The putative *Petunia hybrida* ortholog, AP2α, is capable of complementing ap2 mutants of *Arabidopsis*. In *Petunia*, however, knockout mutations of AP2α did not affect floral organ development, suggesting that AP2 function is redundant in this species [15]. Similarly, two close homologs, LIP1 (LIPESS1) and LIP2, have
been identified in *Antirrhinum majus*, both of which need to be inactivated to get an ap2-like phenotype [16]. Recently, the closest tomato homolog of *AP2, AP2a*, plays a critical role in fruit ripening via regulation of ethylene biosynthesis and signaling [17].

RNA interference (RNAi) is a mechanism of RNA based post-transcriptional gene silencing (PTGS) in eukaryotic cells and has been routinely applied to characterize the gene function and to engineer the novel phenotypes in model plants as well as in cultivated plants. With the recent discovery of gene expression control via small interfering RNA (siRNA) and micro RNA (miRNA) molecules, biologists are exploring genes and development from a new perspective [18]. Understanding of this ubiquitous phenomenon has revealed that RNA interference (RNAi) was a powerful tool to manipulate gene expression and to analyze gene function [19,20]. Gene constructs encoding direct repression induced by ihp-RNA in yeast. Finally, we studied the function of *BnAP2* was required and sufficient for transcriptional activation in *Arabidopsis* using the MEGA5.0 software package, analyzed the expression patterns of *BnAP2* in different organs of *B. napus* by RT-PCR. We had further demonstrated the functional conservation of the *BnAP2* by complementing the ap2-11 mutant of *Arabidopsis* [12]. To study the transcriptional activation of *BnAP2*, we had performed transactivation assays in yeast. Using fusions to the GAL4 DNA binding domain, we showed that the N-terminal of *BnAP2* was required and sufficient for transcriptional activation in yeast. Finally, we studied the function of *BnAP2* using the transgenic approach that involved targeted RNA interference repression induced by ihp-RNA in *B. napus*, floral various phenotype defective and reduced female fertility were observed in *BnAP2-RNAi* lines, loss of the function of *BnAP2* gene also resulted in delayed abscission and senescence of sepal, in the strong *BnAP2-RNAi* lines, seeds showed defects in shape, structure and development and larger size. All the results strongly support the conclusions that *BnAP2* gene is the ortholog of *AtAP2*, and plays a critical role in flower identity and seed development of *B. napus*.

**Results**

**Isolation and sequence analysis of *BnAP2***

Total RNA was extracted from the flower buds of *B. napus* cultivar Zhongshuang 9 and SMART cDNA was synthesized using the purified mRNA as a template. According to the known homologous sequence of *A. thaliana*, the specific primers P1 and P2 (Table S1) were designed and the coding sequence (CDS, 1,299 bp) of *BnAP2* cDNA was obtained from SMART cDNA. The *BnAP2* sequence was submitted to GenBank under the accession number HQ637468.1. The *BnAP2* cDNA encoded a protein of 432 amino acid residues (Fig. 1). Similarity searched using tblastn revealed that the *AtAP2* protein is the most homologous entry in the GenBank database. *BnAP2* showed 86% overall amino acid identity with *AtAP2* and 80% identity with *Brassica rapa AP2* (*BnAP2*). Only full-length sequences with at least 53% amino acid identity were used for further sequence comparison by CLUSTAL X. All possessed high levels of sequence similarity in the double AP2 domain regions (Fig. 1). In addition, the similarity of selected sequences were not limited to the AP2 domains but extended through the reading frame of these genes. A serine-rich putative transcription activation domain (amino acids 16 to 46) [5] and the linker that connected AP2 domains, are conserved, and a putative nuclear localization signal (amino acids 123 to 132) [5] is completely conserved at the amino acid level (Fig. 1). Interestingly, although the relationship of *B. napus* and *B. oleracea* is closer than the other species selected, the levels of sequence similarity of the AP2 domains and the linker are lower (Fig. 1). Nine amino acid substitutions and sixteen amino acids deletions exist in the AP2 domains between *BnAP2* and *BdAP2* genes compared with other *AP2*-like genes, seven amino acids substitutions and 16 deletions of 25 full linker sequences of the predicted occurred (Fig. 1), while the amino acids mentioned above are conserved in the other AP2-like genes. Since *B. napus* is a allotetraploid species resulting from a cross between *B. rapa* and *B. oleracea* [20], these data suggest that the cloned *BnAP2* is the *B. oleracea* copy of *AP2*.

To further evaluate the homology between *BnAP2* and *AP2*-domain gene family in *Arabidopsis*, we compared *BnAP2* with the complete set of *AP2*-domain genes in *Arabidopsis* using the MEGA5.0 software package, analyzed the expression patterns of *BnAP2* in different organs of *B. napus* by RT-PCR. We had further demonstrated the functional conservation of the *BnAP2* by complementing the ap2-11 mutant of *Arabidopsis* [12]. To study the transcriptional activation of *BnAP2*, we had performed transactivation assays in yeast. Using fusions to the GAL4 DNA binding domain, we showed that the N-terminal of *BnAP2* was required and sufficient for transcriptional activation in yeast. Finally, we studied the function of *BnAP2* using the transgenic approach that involved targeted RNA interference repression induced by ihp-RNA in *B. napus*, floral various phenotype defective and reduced female fertility were observed in *BnAP2-RNAi* lines, loss of the function of *BnAP2* gene also resulted in delayed abscission and senescence of sepal, in the strong *BnAP2-RNAi* lines, seeds showed defects in shape, structure and development and larger size. All the results strongly support the conclusions that *BnAP2* gene is the ortholog of *AtAP2*, and plays a critical role in flower identity and seed development of *B. napus*.

**The copy number and expression pattern of *BnAP2***

The copy number of the *BnAP2* gene in the *B. napus* genome was estimated by DNA gel blot analysis. *B. napus* genomic DNA was digested with various restriction endonucleases, fractionated, transferred, and hybridized with the CDS of *BnAP2* cDNA as a probe. As shown in Figure 3A, a small number of bands were observed for each DNA digest, indicating that there may be a few copies of *BnAP2* gene in the genome of *B. napus*.

To monitor the expression pattern of the *BnAP2* gene, we isolated total RNAs from different tissues of *B. napus* plant, such as root, stem, leaf, bud, siliquae (including peel and seed together) and four types of floral organs-sepal, petal, stamen and carpel. By performing reverse transcription-polymerase chain reaction (RT-PCR) analyses with *BnAP2*-specific primer pairs P1 and P3 (Table S1), *BnAP2* gene was clearly expressed at mRNA level in four types of floral organs and thus, functions during the development of all the floral organs (Fig. 3B). Since *AtAP2* is regulated on the protein level by miR172 in whorl 3 and 4, and the miR172 target site is also present in *BnAP2* gene, Western blot analysis of the expression of *BnAP2* protein was performed. And the result revealed that *BnAP2* was expressed at the protein level in whorl 3 and 4 (Fig. 3C). As present, we could not obtain the antibody against *BnAP2* protein, while *BnAP2* showed 86% overall amino acid identity with *AtAP2*, and thus we used *AtAP2* antibody, instead of *BnAP2* antibody in Western blot, RT-PCR analyses with RNAs from root, stem, leaf, bud and siliquae indicated that *BnAP2* gene was expressed in all these tissues, and detected weakly in root, stem and leaf, abundantly in bud and siliquae (Fig. 3D). These results showed that *BnAP2* gene was involved in more global function, in addition to flower identity.
Figure 1. Analysis of the deduced amino acid sequences of BnAP2. Comparison of the deduced amino acid sequences of Brassica napus AP2-like transcriptional factor gene (BnAP2) with other AP2-like genes through CLUSTAL X (1.8) multiple sequence alignment software. Comparison of the overall amino acid sequences, the AP2 domains (shaded), linkers, and putative nuclear localization signals (in boldface) of BnAP2 (GenBank accession...
BnAP2 complements the ap2-11 mutant of Arabidopsis

To further identify BnAP2 as the functional ortholog of AtAP2, we decided to express BnAP2 in the ap2-11 mutant of Arabidopsis to determine whether this might result in complementation. The ap2-11 mutant resulted from an 11-bp deletion in the AP2 gene (bases +724 to +734 relative to the transcription start site, GenBank accession no. U12546), this region encodes the first AP2 domain of the protein. The strong ap2-11 mutant had floral defects similar to those of an ap2 mutant [7,9]. Sepals were transformed into carpeloid organs, petals failed to develop in the second whorl, and stamen number was reduced. The binary vector pBI121-35S BnAP2 was used to transform the ap2-11 mutant (see Methods). All the five independent transformants generated phenotypically wild-type flowers (four sepals, four petals, six stamens) (Fig. 4). Together

Figure 2. Phylogenetic analysis of BnAP2 and the complete set of AP2-domain in Arabidopsis. Phylogenetic relationship was inferred using the Neighbor-Joining method and evolutionary distances were computed using the p-distance method. Assessment of each node confidence was done by means of 1,000 bootstrap replicates. BnAP2 were denoted with black filled circle and At4g36920, the homolog of BnAP2 in A. thaliana with black filled triangle.

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with the sequence and expression data, the complementation data proved that BnAP2 encodes the AtAP2 ortholog in B. napus.

The BnAP2 protein has transcriptional activation activity in yeast

As shown in Figure 1, by comparison with other AP2-like genes, BnAP2 has a serine-rich domain that might act as putative transcription activation domain (amino acids 16 to 46) [5]. To investigate the functional role of this region of BnAP2, we fused the CDS for BnAP2 and its mutants to the GAL4 DNA binding domain expression vector (Fig. 5A) and examined the behavior of each construct as a potential transcriptional activator in yeast (Fig. 5, B and C). In the absence of the GAL4 activation domain, the wild-type BnAP2 protein fused to the GAL4 DNA binding domain activated the transcription of lacZ reporter gene. This result indicated that the BnAP2 protein was capable of functioning as a transcriptional activator in yeast. To identify a minimal transcription activation domain of BnAP2, various deletion mutants of BnAP2 also were tested in the same manner. The BnAP2ΔC1, BnAP2ΔC2, BnAP2ΔC3, BnAP2ΔC4 and BnAP2ΔC5 mutants, which lacked 59, 119, 179, 252, 332 C-terminal amino acids, respectively, showed almost complete β-galactosidase activity (Fig. 5, B and C), while BnAP2ΔN mutant which lacked 100 N-terminal amino acids, demonstrated absence of complete β-galactosidase activity (Fig. 5, B and C), indicating that the N-terminal 100 amino acids of BnAP2 play an important role in supporting the ability of BnAP2 as a potential transcriptional activator.

Generation of the stably inherited BnAP2-RNAi transgenic B. napus plants

We constructed an RNAi vector targeting a 5’ terminal 400-bp cDNA fragment of BnAP2. The resulting construct, designated BnAP2-RNAi, contained the sequence encoding N-terminal transcriptional activation region in an inverted repeat orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter (Fig. 6A). Transformation was performed as the method reported by De Block et al. [29]. Kanamycin-resistant regenerate plants that were phenotypically identical to untransformed plants, rooted well in selective medium, were firstly subjected to PCR-based screening with NPTII gene specific primers P19 and P20 (Table S1). A total of 6 independent BnAP2-RNAi transgenic lines were obtained (Fig. 6B). The transgenic nature for each of them was further confirmed by Southern-blot analysis (Fig. 6C). The results indicated that the transgenic lines BnAP2-RNAi-3, BnAP2-RNAi-4, BnAP2-RNAi-18 and BnAP2-RNAi-26 had a single copy of the transgene, and the transgenic lines BnAP2-RNAi-1 and BnAP2-RNAi-2 had two copies of the transgene. In the second generation, the lines BnAP2-RNAi-3, BnAP2-RNAi-4, BnAP2-RNAi-18 and BnAP2-RNAi-26 segregated in a 3:1 ratio for the transgene as determined by PCR detection. For the lines BnAP2-RNAi-1 and BnAP2-RNAi-2, the ratio was 15:1. From the fourth generation, these lines were no longer segregating for the transgene and thus were considered homozygous. BnAP2 transcripts could not completely be detected in homozygous transgenic lines BnAP2-RNAi-1, BnAP2-RNAi-18 and BnAP2-RNAi-26 by RT-PCR analyses with BnAP2-specific primer pairs, while BnAP2 gene expression was not decreased in homozygous transgenic lines BnAP2-RNAi-2, BnAP2-RNAi-3 and BnAP2-RNAi-4 (Fig. 6D).

Floral patterning defects in BnAP2-RNAi lines

Figure 7 shows representative defective phenotypes of flowers in homozygous BnAP2-RNAi lines. Out of six analyzed transformants, one BnAP2-RNAi line, BnAP2-RNAi-18, shows a milder phenotype (Fig. 7A), and two BnAP2-RNAi lines, BnAP2-RNAi-1 and BnAP2-RNAi-26, exhibit severe alterations in flower identity and development (Fig. 7, B and C), and the other three BnAP2-RNAi lines, BnAP2-RNAi-2, BnAP2-RNAi-3 and BnAP2-RNAi-4, show no obvious floral patterning defects (data not shown).

As for strong floral defects, the sepals in the first whorl were greatly increased in size, both in length and in width, showing distinct leaf-like characteristics, such as a thinner blade in which venation could be easily observed (Fig. 7D). In addition, the number of sepals reduced, some sepals were fused along the margins, curled at the tip and then transformed into carpeloid organs carrying multiple ovules along the margin, in the late arising or distal flowers along the primary inflorescence (Fig. 7E). The second whorl petals also exhibited dramatic defects. The petals were either absent or reduced in number from four in wild type to between one and three organs. Frequently the petals within a single flower were variable in shape, which formed an aberrant bell-like structure (Fig. 7F). The visible phenotypic defects were in the third whorl of the flower where some stamens were frequently converted to filamentous structures (average 1.59 filaments/flower in BnAP2-RNAi-1 and average 1.68 filaments/flower in BnAP2-RNAi-26, Table 1) that resembled stamen filaments, but had no anther-like structure at the distal end of the filament (Fig. 7G). In most cases, the structures of stamens were morphologically normal, but the number reduced. The average number of organs that developed in third whorl positions was 2.34 in the line BnAP2-RNAi-1 and 2.57 in the line BnAP2-RNAi-26 (wild-type average 6 organs), which indicates that there was a frequent failure of organ development in the third whorl of strong BnAP2-RNAi lines (Table 1). Most of the fourth whorl carpel were morphologically abnormal, the size was somewhat larger (Fig. 7H), and
occasionally misshapen and bent carpels were observed in the late arising or distal flowers (Fig. 7I).

In the weak line \textit{BnAP2-RNAi-18}, floral defects only focused on the whorl 2 and 3 floral organs, including that petals were aberrant in number and shape, stamens number was reduced. Table 1 summarized average floral organ number of per flower in above \textit{BnAP2-RNAi} transgenic plants.

Reduced fertility in \textit{BnAP2-RNAi} transgenic plants

\textit{BnAP2-RNAi} also induced other defects in reproductive development. Although flower number on the primary inflorescence of mutant plants was similar to that of wild type, fertility was negatively affected by \textit{BnAP2-RNAi} lines. For example, Table 2 shows that strong \textit{BnAP2-RNAi} mutants produced fewer elongated siliques on the primary inflorescence compared with wild type. Pistils failed to elongate into siliques when ovules within the pistil had not been fertilized to a significant extent [30]. Consistent with this result, average seed number per silique was lower in RNAi lines as compared with that of wild type. The \textit{BnAP2-RNAi} mutants produced fewer seeds than wild type because of defects in fertility caused by reduction in \textit{BnAP2} activity. And the reduced fertility in mutants was most likely a
consequence of defects in pollen or pistils. To determine pollen viability, fertility and quality, pollen test using I2-KI staining solution or aceto-carmine showed that there was no significant difference between results from the two staining methods. The average pollen viability rate of \( BnAP2 \)-RNAi line was 82.76% from I2-KI staining (Fig. 8A) and 81.58% from aceto-carmine staining (Fig. 8B). Pollen germination experiment in vitro showed that pollens from strong \( BnAP2 \)-RNAi line had normal viability (Fig. 8C). Pollen germination on stigma was also confirmed in \( BnAP2 \)-RNAi mutants by observing the germinating pollen tube on the stigmas 4 h after anthesis (Fig. 8D), Moreover, germinating pollen tubes could enter into ovules (Fig. 8E). In reciprocal crosses experiment, male fertility was further assessed using \( BnAP2 \)-RNAi mutants as the pollinator to cross to Zhongshuang 6, and the average number of seeds each silique produced by this cross was normal; female fertility was assessed using \( BnAP2 \)-RNAi mutants as female parent to cross with Zhongshuang 6, the average number seeds each silique produced by this cross was similar to \( BnAP2 \)-RNAi mutants (Table 3). These results showed that knockdown of the expression of \( BnAP2 \) gene had no effect on pollen viability, fertility and quality, and aberrant pistils induced reduced seed set rate in \( BnAP2 \)-RNAi mutants.

Delayed sepal abscission and senescence induced in strong \( BnAP2 \)-RNAi lines

After anthesis occurs in wild-type plants, the sepals, petals, and stamens normally abscise in a short period. As shown in Figure 9A, the floral organs were detached from the siliques in about 3 days after pollination in wild-type \( B. napus \) plants. In contrast, in strong \( BnAP2 \)-RNAi lines, although the petals and stamens normally
abscessed following pollination, the sepals remained attached closely at almost all positions of the inflorescence (Fig. 9A), even during later silique development (Fig. 9B).

Senescence usually occurs synchronously with the abscission process in wild-type plants. Therefore, in some mutants with abscission-delayed genetic modifications, such as the Arabidopsis etr1-1 mutant, the senescence process is also slowed down [31]. We examined the timing of sepal yellowing and the timing of floral organ withering in the wild type and the BnAP2-RNAi lines. Just like etr1-1, the sepals of the BnAP2-RNAi lines showed a blocked progression of senescence compared with the wild type that showed yellowing on second day after pollination and withering in 3 days after pollination (Fig. 9C and D). In contrast, sepals of BnAP2-RNAi lines kept green following pollination, even with the whole progress of silique development (Fig. 9B).

The strong BnAP2-RNAi plants are sensitive to ethylene

Abscission processes have been divided into ethylene-dependent and ethylene-independent types [31]. To test the ethylene sensitivity of strong BnAP2-RNAi plants, a typical “triple-response” assay [32] was used to determine whether strong BnAP2-RNAi plants have normal ethylene perception and response. Seeds germinated vertically in the dark on growth medium supplemented with 5 mM 1-aminoacyclopropane-1-carboxylic acid (ACC), which is the natural precursor of ethylene. The strong BnAP2-RNAi plants displayed similar triple-response morphological changes compared with the wild-type plants, including the inhibition of hypocotyl growth and root elongation, radical swelling of the hypocotyls, and exaggeration of the curvature of the apical hook (Fig. 10). Therefore, the strong BnAP2-RNAi seedlings did not show any deficiency in the perception and response to ethylene.

BnAP2 is involved in seed development

Except floral defects, mild BnAP2-RNAi lines did not visibly exhibit other defects under standard growth conditions. However, we found that the seeds of self-pollinated strong BnAP2-RNAi lines were structurally and developmentally defective by comparing
wild-type and mutant seed. As shown in Figure 11, seeds of strong BnAP2-RNAi lines had defects in shape, size, structure and development. Compared with the wild type, the fully mature seeds from BnAP2-RNAi-1 and BnAP2-RNAi-26 lines had a distorted shape, exhibiting strong indentations resulting in less round seed (Fig. 11A). The surface of the fully mature BnAP2-RNAi seed was bumpy, and frequently, radicels drilled out from mature seeds (Fig. 11B), causing dry seeds to fail in germination (data not shown). Additionally, mature seeds of BnAP2-RNAi-1 line were larger in size than those of wild type (Fig. 11A). However, the abortive seeds in strong BnAP2-RNAi lines were especially interesting, which were abnormal in shape and empty (Fig. 11C). The embryo of abortive seeds was also abnormal in morphology (Fig. 11D). We compared the morphological and developmental changes between abortive and wild-type seeds. Figure 11E shows that there were no obvious changes in total seed protein content in abortive seeds at 10 DAF, 20 DAF and 30 DAF (days after fertilization), and protein accumulation stagnated at 20 DAF, whereas total protein content of wild type seeds increased gradually. Figure 11F-K shows longitudinal sections through cleared seeds at different developmental stage, including at 10 DAF, 20 DAF and 30 DAF, respectively. Strong BnAP2-RNAi and wild-type seeds initially did not display a significantly morphological difference at 10 DAF, suggesting female gametophytes of both genotypes were fertilized with similar efficiencies (Fig. 11, F and I). At 20 DAF, development of BnAP2-RNAi embryos was slower than wild type (Fig. 11, G and J). At 30 DAF, wild-type embryos were essentially at their final size (Fig. 11K), whereas BnAP2-RNAi embryos stopped growing and developing and gradually withered (Fig. 11H).

Discussion

BnAP2 encodes an AtAP2 ortholog

In this study, we reported the isolation and partial characterization of an AP2-like gene from B. napus, here named BnAP2. Sequence analysis and phylogenetic tree analysis showed that the BnAP2 protein was the closest AP2 homolog described so far and the homology between BnAP2 and AP2 extends throughout the whole protein. BnAP2 are broadly expressed in all four types of floral organs and other vegetative different tissues. Moreover, the expression in an ap2-11 mutant background in Arabidopsis resulted in functional complementation. Fusion protein of BnAP2 with GAL4 DNA binding domain strongly activated transcription in yeast, and the transactivating activity was localized to the 100 N-terminal amino acids of BnAP2. Together with the sequence and expression data, the complementation data and transcriptional activity in yeast indicate that BnAP2 encodes an AtAP2 ortholog in B. napus.

Suppression of BnAP2 gene expression in homozygous BnAP2-RNAi lines

Post-transcriptional gene silencing (PTGS) is a widely used gene suppression approach that selectively silences genes in plants and animals [33]. PTGS works through sequence-specific degradation of mRNA through endonucleolytic cleavage followed by exonuclease digestion [34] and is considered to have evolved in plants for protection against pathogenic RNAs [35]. There are increasing reports of constructs specifically designed to express dsRNA in plants, usually in the form of self-complementary hairpin RNA (hpRNA), eliciting a high degree and frequency of PTGS of invading viruses, reporter transgenes, and endogenous genes [21–23,36,37].

Given that B. napus is thought to be a diploidized tetraploid, with many non-repetitive sequences in genome being present in more than two copies, RNAi is expected to be a powerful tool for

Table 1. Average flower organs No. of per flower in BnAP2-RNAi transgenic plants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average No. of Whorl 1</th>
<th>Average No. of Whorl 2</th>
<th>Average No. of Whorl 3</th>
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<td></td>
<td>Sepals</td>
<td>Carpelloid Sepals</td>
<td>Petals</td>
</tr>
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same time, speculated that transgenic silence led to the consequence. At the BnAP2 PCR analysis showed there was no decrease in the mRNA of transgenic lines did not exhibit any morphological changes. RT-in nous target gene were clearly able to generate phenotypic changes defects. These results demonstrated for the first time, to our BnAP2 PCR in other 3 transgenic plants, which indicated that there was foreign PCR-based screen and DNA gel blot confirmed the integration of RNAi lines of floral homeotic gene expression [43,44]. Clearly, floral organ identity [6–8], and the temporal and spatial regulation establishment of the floral meristem [38–42], the specification of controls three critical aspects of flower ontogeny, including the genetic modification of such multiple-copy genes. Using intron-spliced hairpin RNA (ihpRNA) as RNAi construct that targeted BnAP2 gene, we totally obtained 6 transgenic lines. Although PCR-based screen and DNA gel blot confirmed the integration of foreign BnAP2-RNAi fragment into the genome of B. napus, 5 transgenic lines did not exhibit any morphological changes. RT-PCR analysis showed there was no decrease in the mRNA of BnAP2 in the buds of the corresponding RNAi lines. We speculated that transgenic silence led to the consequence. At the same time, BnAP2 gene expression could not be detected by RT-PCR in other 3 transgenic plants, which indicated that there was specific and efficient suppression of target gene. Of which, two BnAP2-RNAi lines showed significant defects in flowers organs and seed development, and one BnAP2-RNAi line only showed floral defects. These results demonstrated for the first time, to our knowledge, that ihpRNA constructs targeted against an endogenous target gene were clearly able to generate phenotypic changes in B. napus, making this approach an efficient technique for genetic modification of important agronomic traits in oil crops.

Floral defects and reduced fertility induced in BnAP2-RNAi lines

In Arabidopsis, the floral homeotic gene APETALA2 (AP2) controls three critical aspects of flower ontogeny, including the establishment of the floral meristem [38–42], the specification of floral organ identity [6–8], and the temporal and spatial regulation of floral homeotic gene expression [43,44]. Clearly, AP2 plays a critical role in the regulation of Arabidopsis flower development. Null mutants of the AP2 gene in Arabidopsis confer a mutant phenotype that fit almost perfectly with the A-function in the ABC-model. The phenotypes described in the strong RNAi lines are in agreement with the notion that BnAP2 exert the A-function in B. napus. In this study, floral phenotype defectives in B. napus BnAP2-RNAi lines were observed, which was similar to those described in Arabidopsis. Weak BnAP2-RNAi line, having defects in petals and stamens, showed normal fertility. By contrast, two strong BnAP2-RNAi lines not only exhibited the loss of floral identity but also reduced fertility. Subsequent pollen stainability, pollen germination and reciprocal cross experiment indicated that pollens from strong BnAP2-RNAi lines had normal quality and viability. These results told us that knockdown of BnAP2 gene had no effect on pollen fertility and induced aberrant pistils maybe had a negative effect on female fertility. Nevertheless, we could not exclude the possibility that reduced fertility of the RNAi lines resulted from the suppression of other similar AP2 genes, in addition to the BnAP2 gene.

BnAP2 gene is involved in floral organ abscission and senescence

In plants, abscission and senescence events allow the shedding of leaves, flowers, fruits and seeds, and can facilitate growth, reproduction, and defense against pathogens [45,46]. As with most developmental events, proper timing and spacing are crucial during organ separation [47]. Floral organ abscission is a

**Table 2. Fertility and seed weight analysis of BnAP2-RNAi plants.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Flower No.</th>
<th>Elongated Silique No.</th>
<th>Length Per Silique (cm)</th>
<th>Seed No. Per Silique</th>
<th>Seed Weight*(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>42±2.7</td>
<td>42±2.7</td>
<td>11.8±5.3</td>
<td>20±2.3</td>
<td>186</td>
</tr>
<tr>
<td>BnAP2-RNAi-18</td>
<td>45±1.8</td>
<td>40±3.2</td>
<td>10.0±4.2</td>
<td>18±3.4</td>
<td>185</td>
</tr>
<tr>
<td>BnAP2-RNAi-1</td>
<td>50±5.2</td>
<td>8±3.4</td>
<td>4.0±1.6</td>
<td>6±3.8</td>
<td>274</td>
</tr>
<tr>
<td>BnAP2-RNAi-26</td>
<td>48±7.3</td>
<td>10±5.1</td>
<td>5.6±1.2</td>
<td>5±2.4</td>
<td>279</td>
</tr>
</tbody>
</table>

All values pertain to the primary inflorescence. Plants were grown concurrently under identical conditions. Similar results were obtained in an independent experiment that was performed in a different season of the year. Means ± SD are shown.

*Weight of seeds produced on primary inflorescence is given in mg per 50 seeds. Seed weight values that differ at the 0.05 significance level are labeled with A and B letters. Seed weights for wild type and BnAP2-RNAi-18 and values for BnAP2-RNAi-1 and BnAP2-RNAi-26 are not significantly different, respectively.

doii:10.1371/journal.pone.0033890.t002

**Table 3. Aberrant pistils leads to reduced fertility in strong BnAP2-RNAi plants.**

<table>
<thead>
<tr>
<th>Parent</th>
<th>Flower No.</th>
<th>Elongated Silique No.</th>
<th>Length Per Silique (cm)</th>
<th>Seed No. Per Silique</th>
<th>Seed Weight*(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>10</td>
<td>10</td>
<td>12±3.3</td>
<td>19±3.1</td>
</tr>
<tr>
<td>BnAP2-RNAi-1</td>
<td>BnAP2-RNAi-1</td>
<td>10</td>
<td>10</td>
<td>11.6±3.3</td>
<td>20±1.1</td>
</tr>
<tr>
<td>BnAP2-RNAi-26</td>
<td>BnAP2-RNAi-1</td>
<td>10</td>
<td>10</td>
<td>11.4±4.5</td>
<td>21±4.2</td>
</tr>
<tr>
<td>BnAP2-RNAi-1</td>
<td>BnAP2-RNAi-1</td>
<td>10</td>
<td>3</td>
<td>6±2.6</td>
<td>7±3.5</td>
</tr>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>10</td>
<td>4</td>
<td>5.8±1.3</td>
<td>3±2.6</td>
</tr>
<tr>
<td>BnAP2-RNAi-26</td>
<td>BnAP2-RNAi-26</td>
<td>10</td>
<td>3</td>
<td>6±1.8</td>
<td>4±2.7</td>
</tr>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>10</td>
<td>4</td>
<td>5±3.2</td>
<td>4.5±1.5</td>
</tr>
</tbody>
</table>

Reciprocal crosses between wild-type plants and strong ap2 mutant plants were performed on secondary inflorescences. Plants were grown together in the same conditions. Three flowers at identical positions (11th to 13th flowers for wild type or male-sterile mutant, 10th to 15th flowers in ap2 mutants) were manually pollinated. Two to four inflorescences per plant and four to five plants were used for calculations. Similar results were obtained in an independent experiment that was performed in a different season of the year. Means ± SD are shown.

doii:10.1371/journal.pone.0033890.t003
in contrast, sepals of strong BnAP2

day following pollination, sepals of the wild type were detached, in

vertical half-strength Murashige and Skoog (MS) agar plates in the

BnAP2 growing seedlings of wild-type and strong BnAP2

Figure 10. Triple response phenotype of wild-type and strong

Figure 9. Senescence and abscission were delayed in strong

BnAP2-RNAi lines. A, Floral organs abscission along the inflorescence

in the wild type (WT) (right) and BnAP2-RNAi lines (left). B, Sepals in

BnAP2-RNAi lines retained with silique development. C, On second day

following pollination, sepals of the wild type demonstrated yellowing,
in contrast, sepals of strong BnAP2-RNAi lines keep green. D, On third
day following pollination, sepals of the wild type were detached, in

contrast, sepals of strong BnAP2-RNAi lines remained. doi:10.1371/journal.pone.0033890.g009

developmentally controlled program that occurs after flower

pollination. Ethylene is considered to be a fundamental regulator

of the abscission rate [31,48]. Defects in the components of the

ethylene perception and signaling pathways will delay abscission to

various degrees. In Arabidopsis ethylene-insensitive mutants, such as

etr1 and ein2, both floral organ abscission and senescence are

delayed [31,46]. However, abscission processes have been divided

into ethylene-dependent and ethylene-independent types [31], and

therefore further experiments would be carried to determine

whether the BnAP2-RNAi plants have normal ethylene perception

and response.

However, ethylene is not the only regulator of abscission, and

abscission processes have been divided into ethylene-dependent

and ethylene-independent types [31]. A ligand gene family,

including INFLORESCENCE DEFICIENT IN ABSCISSION

(IDA) and five IDALIKE (IDL) genes, participates in the control of

abscission, and its function is not affected by exogenous ethylene

[49,50]. In addition, some genes that relate leaf and floral organ

patterning also influence the capacity for abscission. These genes

include two well-known leaf-patterning factors, BOP1/BOP2 [51],
a MADS box domain transcription factor, AGL15, and two

chromatin regulators, ARP4 and ARP7 [52–54]. The triple-

response assay of wild type and BnAP2-RNAi-26 plants confirmed

that the BnAP2-RNAi plants had normal ethylene perception

and response. And the result suggested that BnAP2 gene had an effect

on sepal abscission with the ethylene-independent pathway. At the

same time, we could not exclude the possibility that delayed sepal

abscission and senescence of the RNAi lines was caused by the

inhibition of other similar AP2 genes, in addition to the BnAP2

gene.

Knockdown of BnAP2 gene expression disordered seed development

Our analysis of BnAP2 gene expression at the RNA level revealed

that BnAP2 was expressed in both nonfloral and floral tissues and organs. We therefore proposed that BnAP2 had a more expanded role in B. napus growth and development. In strong B. napus BnAP2-RNAi lines, dried, full and mature seeds produced through self-pollinating were larger and more variable in shape than circle-formed wild-type seeds because of knockdown of BnAP2 gene. In addition, some full and mature seed coat development was affected, leading to the radicles exposed to seed coat.

Seed development in higher plants begins with a double fertilization process that occurs within the ovule and ends with a dormant seed primed to become the next plant generation [55,56]. Many transcription factors (TFs) are responsible for controlling seed development. Le et al. [57] carried global analysis of gene activity during Arabidopsis seed development and identified 289 seed-specific genes, including 48 that encode TFs. In Arabidopsis, AP2 was involved in controlling ovule, seed coat development, seed size and seed development [10–12,41,58]. Then, these results suggested that BnAP2 gene played an important role in seed development.

Study of abortive seed development in B. napus BnAP2-RNAi

lines further confirmed above proposal. The seed consists of three

major compartments, the embryo, endosperm and seed coat, that

originate from different cells of the ovule and possess different

complements of maternal and paternal genomes [59]. Seed

development proceeds through two distinct phases during which

growth of the three compartments is coordinated. During the early

morphogenesis phase, the embryo undergoes a series of differenti-

tation events in which the plant body plan is established with the

formation of embryonic tissue and organ systems. It is also during

this phase that the endosperm mother cell initially undergoes

nuclear division without cytokinesis to form a syncytium [60].

Syncytial nuclei are sequestered into individual endosperm cells

through the process of cellularization, and the endosperm

continues to grow through periclinal cell divisions at the periphery

of the endosperm. Later in embryogenesis during the maturation
phase, the embryo and endosperm accumulate reserves such as storage lipids and proteins, and the embryo acquires the ability to withstand desiccation [61,62]. In plants such as *B. napus*, the endosperm is largely consumed by the developing embryo such that only a few endosperm cell layers remain in the mature seed. In *B. napus* *BnAP2*-RNAi lines, total protein content of abortive seeds showed no conspicuous alterations at 10 DAF, 20 DAF and 30 DAF. This result suggested that there was an obstacle in accumulating reserves such as proteins in embryogenesis during the maturation phase. Frozen section analysis revealed that during the early morphogenesis phase of abortive seeds, the embryo successfully undergoes a series of differentiation events in which the plant body plan was established with the formation of embryonic tissue and organ systems. But embryos established did not successfully develop into normal and full seeds, stopping growing at 20 DAF. Together, loss of *BnAP2* function influenced seed development in embryogenesis during the maturation phase.

In conclusion, as a key transcriptional factor, *BnAP2* gene is involved in the regulation of many target genes and plays essential roles in floral identity, sepal abscission and senescence, and seed development in *B. napus*. Since the RNAi lines, in addition to the *BnAP2* gene, also affected the expression of other similar AP2 genes, we could not exclude the possibility that reduced fertility and delayed sepal abscission and senescence was led by the suppression of other paralogues. And further experiment would be conducted in order to screen paralogues affected in the RNAi lines through transcriptome profiling. Although knockdown of *BnAP2* gene caused mature and full seeds to be larger than wild seeds, total seed yield could not be raised in *BnAP2*-RNAi lines because of reduced female fertility and abortive seed. However, it is unclear how *BnAP2* acts to affect seed development. Additional experiments are needed to understand how *BnAP2* affects seed development and seed size at a mechanistic level. To unravel the genes that might be directly or indirectly regulated by *BnAP2* in *B. napus*, further research would be focused on comparing gene expression of seed development between wild type and transgenic knockdown plants through transcriptome profiling.

**Materials and Methods**

**Isolation and sequencing of the *BnAp2* gene**

Total RNA was extracted from the flower buds of *B. napus* cultivar Zhongshuang 9 using the RNeasy Plant Mini Kit (Qiagen, USA) and first-strand cDNA was synthesized following the procedure described by SMARTâ"“PCR cDNA Synthesis Kit (Clontech, TaKaRa) and was used as a template. Two specific

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*Figure 11. Strong *BnAP2*-RNAi lines cause changes in seed development.*** A, Seeds of *BnAP2*-RNAi lines are less round in shape and larger in size than that of wild type (left, *BnAP2*-RNAi; right, wild type). B, The surface of the full *BnAP2*-RNAi seed is bumpy and radicels drill out from mature seeds. C, Abortive seeds in strong *BnAP2*-RNAi lines (arrow). D, Embryo of abortive seeds (left, *BnAP2*-RNAi; right, wild type). E, *BnAP2*-RNAi-26 plant seeds contain less protein than do wild-type seeds. Protein extracts from an equal number of wild-type and *BnAP2*-RNAi-26 seeds were fractionated on a 12% SDS polyacrylamide gel and stained. Molecular mass markers are shown to the left of the gel. Optical sections through developing *BnAP2*-RNAi (F–H) and wild-type seeds (I–K) at the indicated DAP. Seeds from three to five siliques at each time point were harvested, fixed and cleared and representative optical sections are shown. doi:10.1371/journal.pone.0033890.g011*
primers P1 and P2 (Table S1) were designed based on the known AP2 gene sequence of *A. thaliana*. PCR reaction was performed as follows, firstly denatured at 94°C for 2 min, then 94°C 30 s, 64°C 30 s, 72°C 90 s for 14 cycles and the annealing temperature decreased 1°C every cycle, then 94°C 30 s, 50°C 30 s, 72°C 90 s for 22 cycles, and then 72°C 5 min. The specific fragment obtained was cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced at Beijing Huada Company (China).

RT-PCR

Total RNA was isolated from root, shoot, leaf, flower bud, silique (including peel and seed) and four types of floral organs using the RNeasy Plant Mini Kit (Qiagen, USA), respectively. And then, RNA was treated with DNase, extracted with phenol/ chloroform, and precipitated, and 0.5 μg of total RNA was reverse transcribed from an oligo-dT primer by using the SuperScript® III First-Strand Synthesis System (Invitrogen). The first-strand cDNA procedures described by DIG High Prime DNA Labeling and hybridization and detection were performed following the Vector construction.

Gene sequence of *AP2* with appropriate restriction enzyme. The restriction fragments specific to the *BnAP2* gene were amplified a 756-bp product. For Southern blot analysis promoter probe were P21 and P22 (Supplemental Table S1), which confirmed the integration of the target gene, the probe was the method reported by Yu et al. [64]. For Southern blot analysis to give products of 600 bp. PCR analysis was done according to the method described previously [29]. Kanamycin-resistant plantlets that rooted well in selective medium were transferred to pots and grown in a glasshouse. For plants grown in a plant growth room, the growth conditions were 20±2°C under a 16/8 h photoperiod at a light intensity of 44 μmol m−2 s−1 and 60-90% relative humidity. For plants sown in a glasshouse, the conditions were 14-22°C and natural light.

Ethylene response

Ethylene treatment was performed as described previously [68]. For the triple response, 5 mM of the ethylene precursor ACC was added to Murashige and Skoog (MS) medium for germination of wild-type or transgenic seeds in the dark for 3 d.

Transactivation activity assays

For the yeast strains and transformation, the yeast strain *Saccharomyces cerevisiae* strain AH109 (MATa, HIS3, IacZ, ADE2, MEL1) was transformed with plasmids containing CDS of *BnAP2* cDNA or *BnAP2* fragments which were fused in frame to the yeast GAL4 DNA binding domain vector pGBKKT7, using a modified lithium acetate procedure [69]. Transformants were selected on plates containing synthetic medium lacking tryptophan. The colony lift filter assay and liquid culture assay using o-d-galactopyranoside as a substrate were performed subsequently as described by the manufacturer (Clontech, Palo Alto, CA) to determine the ability of each translation product to activate transcription.

For the filter lift β-glucosidase assay, transformants were streaked onto a synthetic complete supplement (SC)-Trp-His-ADE medium, grown at 30°C for 3 d, and lifted from the medium using filter papers (Whatman No. 5). The filters were immersed into liquid nitrogen for 30 s, thawed at room temperature for 5 min, and placed on filter papers soaked with an X-gal (0.5 mg/ml) solution. The reactions were stopped after 2 to 4 hours at 30°C. For each construct, 5 independent transformants were assayed.

**Plant transformation (Arabidopsis complement experiment, B. napus transformation)**

For *Arabidopsis* transformation, the *Arabidopsis ap2-11* mutant (kindly provided by John J. Harada) was transformed according to the vacuum infiltration method [67] using *Agrobacterium* strain EHA105. T3- or T4-generation homozygous plants were used for phenotype analysis.

The system was used in this study. The plasmid pBnAP2AN1 encoded *BnAP2* proteins with deletion of 100 N-terminal amino acids. The plasmid pBnAP2 which encoded full *BnAP2* protein was generated by PCR with P6 and P14.

**Vector construction**

*BnAP2* gene over-expressing vector used to transform *Arabidopsis* was constructed by PCR using the synthetic oligonucleotide primers P6 and P7 (Supplemental Table S1). The amplified full-length *BnAP2* cDNA fragment was digested with *Bgl*II, then cloned into pBI121 [65], which had been cut with *Bam*HI. The resulting *BnAP2*-expressing vector was designated as pBI121- *BnAP2*.

Vector construction for RNAi knockout, RNAi vector pHANNIBAL [23] was used to generate RNAi constructs. The sense and antisense cDNA sequences of the *BnAP2* gene were amplified from CDS of *BnAP2* cDNA through PCR and placed under the control of a constitutively expressing 35S promoter in pHANNIBAL. The sense fragment was amplified using gene specific primers having restriction sites *Eco*R1 (P15) and *Kpn*I (P16) and inserted as an *Eco*R1-*Kpn*I fragment into pHANNIBAL which was cut with *Eco*R1 and *Kpn*I (Supplemental Table S1). The antisense fragment was amplified using gene-specific primers having restriction sites *Xho*I (P17) and *Bgl*III (P16) and inserted as an inverted fragment as *Xho*I-*Bgl*III pHANNIBAL which was cut with *Xho*I and *Bam*HI (Supplemental Table S1). The pHANNIBAL vector was then subcloned at *Nde*I sites into a binary vector pART27 [66]. For construction of various *BnAP2* deletion mutants and full *BnAP2* for transactivation activity assays, the deletion mutant genes were generated by PCR using restriction sites within the synthetic oligonucleotide primers. The primers were shown in Table S1. The forward primers carried *Eco*R1 and the reverse primers had *Bgl*III. The restriction sites used in the primers to facilitate the cloning of the PCR product into the yeast GALA DNA binding domain vector pGBK17 were underlined. The plasmids pHbAP2AC1, pHbAP2AC2, pHbAP2AC3, pHbAP2AC4 and pHbAP2AC5, encoded *BnAP2* proteins with deletion of 59, 119, 179, 292 and 332 C-terminal amino acids, respectively. The plasmid pHbAP2AN1 encoded *BnAP2* proteins with deletion of 100 N-terminal amino acids. The plasmid pHbAP2 which encoded full *BnAP2* protein was generated by PCR with P6 and P14.
For the liquid β-galactosidase assay, colonies of yeast transformants were grown in an SC-TT-p-His-ADE medium to an A₆₀₀ of approximately 0.5. The cultures were diluted 4 times with fresh media and grown further for 3 h. After the A₆₀₀ was measured, 1.5-ml aliquots of the cultures briefly underwent centrifugation in a microfuge. The pellets were resuspended in 0.3 ml of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄·7H₂O) without β-mercaptoethanol, microfuged briefly again, and resuspended in 0.3 ml of Z-buffer. One third of the suspension was transferred to a fresh tube, placed in liquid nitrogen until frozen, and thawed in a 37°C water bath. The freeze-thaw cycle was repeated 2 more times. Afterward, 0.7 ml of Z-buffer with β-mercaptoethanol and 0.16 ml of o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml in Z-buffer) were added to start the reaction. Incubation was continued at 30°C until the color changed to yellow. Reactions were stopped by the addition of 0.4 ml of 1 M Na₂CO₃. The mixtures underwent microcentrifugation for 10 min to remove cell debris, and the A₆₀₀ was measured. Four independent transformants were assayed for each construct, and the β-galactosidase activity was expressed in Miller units.

**Determination of pollen viability, fertility and quality**

Pollen viability test was performed with the use of two staining procedures, one reported by Shinjyo [70] and the other by Wei et al. [71], and in vitro pollen germination. Newly opened flowers were sampled at 9-10 a.m. Anthers were squashed and pollen grains were stained with 1% w/v iodine/potassium iodide solution [I2-KI] to observe starch accumulation or with 1% aceto-carmine, and were observed under microscopy. Five individual plants of each material and five flowers of each plant were used and the calculation was based on 200 pollen grains of each flower. Pollen viability rate was calculated as the number of well-stained pollen grains/total pollen grains ×100%. For pollen germination in vitro, pollen grains was isolated from flowers of strong BnaAP2-RNAi lines and immediately germinated in vitro using Hodgkins and Lyons media containing 9% sucrose and 13% polyethylene glycol (MW 4000) [72]. The pollen was incubated in light and high humidity for 6 h at 23°C. Germinating pollen (those with pollen tubes greater than twice the length of the pollen grain) were counted and photographed. Pollen germination on the stigmas was observed using aniline blue staining as previously described [73]. Briefly, 3 h after pollination, pistils were fixed with ethanol/acetic acid (3:1 v/v) for 30 min, washed with 1 N KOH at 55°C for 30 min, and stained with 1% w/v aniline blue for 40 min at 37°C. Pollen fertility of BnaAP2 RNAi mutants was also assessed according to reciprocal crossing between Zhonghuang 6 wild-type plants and BnaAP2-RNAi mutants. Plants were grown together in the same conditions. Flowers at identical positions were manually pollinated. Two to four inflorescences per plant and four to five plants were used for calculations. Similar results were obtained in an independent experiment.

**Protein analysis**

Ten seeds from Zhonghuang 6 wild-type plants or BnaAP2-RNAi lines, at 10 DAY, 20 DAY, 30 DAY, respectively, were homogenized with 100 ul of extraction buffer [74] by using a microglass pestle and mortar, respectively. After centrifugation, 10 ul of each extract was used for SDS-PAGE [75]. Protein content in 5 µl of each extract was determined by using the Bio-Rad RC-DC Protein Assay Kit with BSA as the standard.

**Western blot analysis**

Protein extracts of flower buds, floral organs in whirl 3 and 4, respectively, were prepared according to the method described above. The concentration of protein in the supernatant was quantified using a protein assay kit (Bio-Rad). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes were probed with a goat polyclonal AtAP2 antibody (Santa Cruz), followed by incubation with horseradish peroxidase conjugated donkey anti-goat IgG (Santa Cruz). Immunoreactivity was visualized by chemiluminescent detection (Pierce). Immunoblots were visualized with the ECL detection system (Pierce).

**Phylogenetic Analysis**

To detect BnAP2 homolog in A. thaliana, all the protein sequences of AP2-domain genes in A. thaliana were downloaded from RARTF database [http://rarge.psc.riken.jp/rartf/] [76]. Selected 145 Arabidopsis AP2-domain genes and target BnAP2 were used to constructed phylogenetic trees with the MEGA5.0 software package [77–80]. Phylogenetic relationship was inferred using the Neighbor-Joining method and evolutionary distances were computed using the p-distance method.

**Frozen section**

Seeds at different developmental phase were fixed with FAA (10% formalin/5% acetic acid/45% ethanol/0.01% Triton X-100) for 45 min, and rehydrated through an ethanol series. Seeds were then embedded in OCT compound and frozen, sectioned at 10-μm thickness with frozen section machine Leica CM1850 (Germany). Observations were made with an Olympus microscope and photographed.

**Supporting Information**

Table S1: List of primers used. (PDF)

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Thanks to Prof. John J. Harada (Section of Plant Biology, Division of Biological Sciences, University of California, One Shields Avenue, Davis, CA 95616) for providing us with the ap2-11 mutant of Arabidopsis.

**Author Contributions**

Conceived and designed the experiments: WW XY. Performed the experiments: XY LZ BC. Analyzed the data: ZX CC JY. Contributed reagents/materials/analysis tools: LW CL WW. Wrote the paper: XY CL WW.

**References**


