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Overexpression of *CnANT*, Coconut *BABYBOOM* Homologue Alters Plant Growth and Morphology in Transgenic *Arabidopsis* Plants

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ABSTRACT. *Identification of molecular markers for the recognition of tissues competent for in vitro regeneration has a considerable potential for increasing the efficiency of coconut somatic embryogenesis protocol. BABYBOOM (BBM), APETALA2/Ethylene Responsive Element Binding Protein (AP2/EREBP) family gene has shown a role during embryogenesis. To gain insight into the functions of CnANT, a BABYBOOM gene homologue of coconut was cloned under the cauliflower mosaic virus-35s promoter and the plant growth and developmental changes that occur in transgenic Arabidopsis plants were determined and compared with the data obtained for other BBM genes originated from other plant species. Construction of vectors for Arabidopsis transformation was based on pGREEN plasmid. Transformed plants were screened by spraying herbicide Harvest which contains the active ingredient gluphosinate ammonium. Five independent transgenic lines were developed. All lines showed the typical 3:1 segregation ratio suggesting that the bar genes had integrated into the Arabidopsis as a single segregating locus. In comparison to wild type plants, overexpression of CnANT resulted in pleiotropic phenotypes including compact rosettes, backward curly-leaves with serrated margins, larger flowers and smaller siliques. There were also modifications in plant growth such as delay in flowering. Similar phenotypic effects observed for CnANT along with BBM and other related genes from eudicots suggest similar biochemical targets governed by these proteins. Similarity of their expression, thus suggest that CnANT gene also has a potential to increase in vitro regeneration response.*

Key words: *Coconut, CnANT, Arabidopsis, transgenic*

INTRODUCTION

Among the genes involved in embryogenesis, APETALA2 family genes specially from AINTEGUMENTA (ANT) sub group, play an important role in both early and late phases of development (Boutilier *et al.,* 2002). Amongst them, *BABY BOOM* which was cloned from *Brassica napus* (*BnBBM*) induced somatic embryos in transgenic *Arabidopsis* cotyledons when ectopically over-expressed (Boutilier *et al.,* 2002). Heterologous expression of *BBM* in tobacco induced spontaneous callus and shoot formation (Srinivasan *et al.,* 2007). Together with the enhanced tissue response for in vitro culture, these genes have caused several morphological and developmental changes in transgenic plants. Furthermore, *ANT* genes appears to be involved in cell proliferation in many organs and differentiation of stem cell niches within meristems (Nole-Wilson *et al.,* 2005). In a recent study, oil palm

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AINTEGUMENTA-like gene *EgAP2-1* in transgenic *Arabidopsis* plants showed phenotypic alterations and enhanced the *in vitro* regeneration capacity (Morcillo *et al.,* 2007).

The ability to produce transgenic plants using successful *Agrobacterium tumefaciens*-mediated transformation has greatly accelerated the study of functional genomics. There are several advantages in *Agrobacterium*-based transformation technology over other DNA transformation methods. It generally produces a higher frequency of single copy transgenic plants which is one of the most important factors in molecular studies (Dai *et al.,* 2001). Further, *Agrobacterium* mediated transformation offers an opportunity to produce lines free from selectable marker genes (Qiu *et al.,* 2010). Transgenic plants are normally produced using methods that require additional sample preparation steps and tissue culture practises (Christou 1996; Hooykaas and Schilperoort 1992). However, *Agrobacterium* 'vacuum filtration' method which was developed for *Arabidopsis* transformation (Bechtold *et al.,* 1993) bypassed these critical time consuming steps, making it popular for transformation experiments. 'Floral dip' later on offered a substantially, simplified protocol for *Arabidopsis* transformation by eliminating some of the components of the infiltration method such as laborious vacuum infiltration process and the uprooting and re-planting of plants during application of *Agrobacterium* (Clough and Bent 1998). Over expression studies using *Agrobacterium* transformation have been extensively used to study the effect of several embryogenesis related genes from a range of monocot and dicot plant species in *A. thaliana* (Morcillo et al., 2007; Alemanno *et al*., 2008; Ouakfaoui *et al*., 2010).

With the goal to explore the possible application of *ANT* genes as markers for the development of *in vitro* culture of coconut, *CnANT* gene, an Arabidopsis *BBM* ortholog, was cloned under the cauliflower mosaic virus-35S promoter in transgenic Arabidopsis plants and its effect on plant growth and development were studied.

MATERIALS AND METHODS

Plant material

A. thaliana ecotype Columbia (Col-0) was taken as the wild type. After 3 days from stratification, seeds were planted in individual pots (8 cm diameter) containing seed sowing compost and maintained in a controlled environment cabinet (Saxil or Fisons) at 25°C with LD conditions (16 h light).

Plasmid construction

The full length cDNA of *CnANT* which encodes an AP2 transcription factor protein comprising 474 amino acids was cloned in to TOPO vector (Invitrogen, UK). This was amplified using the primers modified to contain specific recognition sites for *XbaI* and *BamHI* restriction enzymes [Forward primer (CnANTrF1): 5'-ATA TAT TCT AGA ATG GAC ATG GAC GCT TCA C-3', Reverse primer (CnANTrR2): 5'-ATA TAT GGA TCC TTC CCA CCC ATC CAT ATC AT-3' (Underlined are the restriction recognition sites)]. Construction of vectors for *Arabidopsis* transformation was based on pGREEN plasmid. pGreen II 0229-2ab plasmid modified to have a 35S expression cassette (Ordidge *et al.,* 2005) was digested with *XbaI* and *BamHI*, purified by agarose gel electrophorasis and ligated the amplified *CnANT* DNA using quick-stick ligation kit (Bioline, UK) according to the manufacturer's protocol. Plasmids were transformed into DH 5α cells and plated on LB plates containing 50 mg/L kanamycin. Positive colonies were screened by PCR using 35S

primers. These were further confirmed by sequencing and restriction digestion of isolated plasmids. The plasmid was designated pG0229bCnANT (Figure 1).

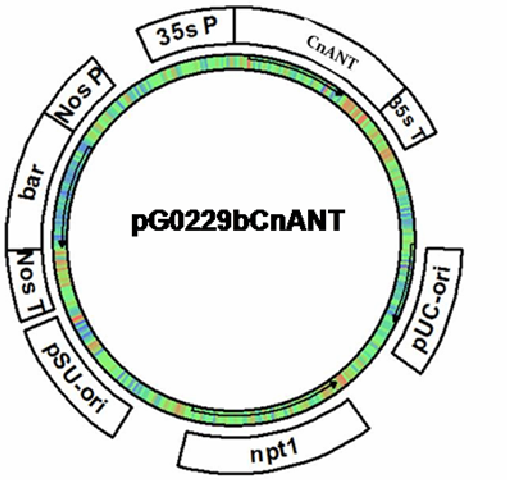

Fig. 1. Structure of the pG0229bCnANT plasmid.

Kanamycin resistance is conveyed by npt1; bar grants resistance to glufosinate ammonium; *CnANT* is the gene of interest; UC-ori and pSU-ori are origins of replication.

Agrobacterium transformation

One micro litre each of pG0229bCnANT (hereafter mentioned as 35S:*CnANT*) (50-200 ng) and pSOUP (50 ng) plasmid mini preparations (Qiagen) were mixed with thawed electro competent *Agrobacterium* cells (40.0 µL). The mixture was placed in a pre-chilled 2 mm gap electroporation cuvette (Molecular BioProducts, UK). Transformation was achieved by giving an electric pulse using a Bio-Rad gene pulser (BioRAD, USA) with the settings at 2.50 kV, 25 uFD and 400 Ω. One ml of fresh LB broth was added to the cuvette and cells were allowed to recover for 2 h at 28°C with shaking at 250 rpm. Finally the cells were plated (10.0 µL and 100.0 µL volumes) on LB plates with 50 mg/L kanamycin and incubated at 28°C for 4 d. Positive colonies were screened by colony PCR using 35S primers, sub-cultured and glycerol stocks were prepared for long term storage at -80°C.

Agrobacterium mediated transformation of *A. thaliana*

A. thaliana plants were transformed by the floral dip method as described by Clough and Bent (1998). *A. thaliana* plants were grown under LD conditions until they flower. When all plants were bolted, the first inflorescence axis was removed to enhance the synchronous production of secondary inflorescences. For transformation, plants were dipped with the *Agrobacterium* solution when most of the secondary inflorescences were of 10 cm height which was achieved after 5-8 days after removing first inflorescence. *Agrobacterium* which harboured 35:*CnANT* and pSOUP was centrifuged at 5500 g for 20 min at room temperature, re-suspended in 5% sucrose supplemented with 500 µL/L Silvet 77 (OSi Specialties Inc, USA) to obtain 0.8 optical density at 600 nm, and used for transformation. Two hundred millilitres of the *Agrobacterium* solution was placed in a 250 mL plastic bottle and inflorescences were dipped carefully for 3-5 s. Plants were then placed in the dark and

covered with polypropylene bags for 12 h. Uncovered plants were transferred to LD conditions as mentioned previously and allowed to grow until pods ripened. Seeds were harvested and stored at room temperature (approximately 23 °C).

Screening of transformants

Cold treated seeds were grown on seed sowing compost in 6" X 8" plastic trays as described previously. The seedlings were sprayed with gluphosinate ammonium (Harvest, Bayer Crop Science, UK) at a concentration of 150 mg/L. The seedlings were sprayed further two times to screen any late germinated seeds. The surviving plants (T_1 plants) were planted on individual pots and maintained at standard conditions as described earlier. Seeds (T_2) of these T_1 plants were harvested and stored at room temperature (approximately 23 °C).

Detection of transgenic plants by PCR

Genomic DNA from leaf material was isolated from the primary transformants and analyzed by PCR. Primer were from the 35S cassette (forward: 5'- CTA CAA ATG CCA TCA TTG -3'; reverse: 5'-CCC TAA TTT CCC TTA TCG GG-3') to amplify ~ 1800 bp fragment when the correct insert of *CnANT* gene was present. The PCR parameters were one cycle at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s. The final extension was at 72 ^{o}C for 10 min.

Production of transgenic lines

T_2 seeds were obtained allowing self pollination of primary transformed *A. thaliana* plants (T_1). For the segregation analysis, 50 randomly selected seeds were germinated on seed sowing compost in a 6" X 8" plastic tray and screened with gluphosinate ammonium. The number of surviving plants were counted (T_2 plants), planted in individual pots and maintained at standard conditions. The plants were allowed to self pollinate and seeds were collected. T_3 Seeds of each T_2 plant (25 seeds per plant) were germinated, screened and maintained as described previously.

Morphological evaluation of transgenic plants

The morphological characters of transgenic and control plants were evaluated on plants grown under controlled environment. A randomized complete design (RCD) with eight replications was used. Types of data recorded are summarized in Table 1. Flower buds from transgenic and control *Arabidopsis* plants were collected for studying the pollen viability. For each line flowers were collected from two individuals. Unopened flower buds were dissected under stereo binocular microscope. Twelve anthers from two florets were squashed in a drop of 4 % (v/v) aceto-carmine on a slide. A cover slip was placed on the squashed material and examined under light microscope.

Table1. Summary of morphological data recorded in 35S:*CnANT* transgenic*Arabidopsis* plants.

Trait	Data recorded
Number of days to flower	The number of days since seeds were sown in MS plates to bolting when the primary inflorescence reached 1 cm height.
Sillque size	Lengths of ten siliques from the main inflorescence of each plant in a particular line were measured starting from the bottom.
Sterile pods (empty pods)	Number of empty pods were counted in 5 secondary inflorescences per plant starting from the top of the inflorescence up to 24 pods
Leaf number	Number of leaves at the time of flowering was recorded for each individual in a line.
Secondary inflorescence number	Number of secondary inflorescences at seed maturity stage was counted

Statistical analysis

Statistical analysis was performed using Genestat 13th edition. One way ANOVA was performed on data of morphological characters of transgenic and wild type *Arabidopsis* followed by Least Significant Difference test to determine the different performances of transgenic lines. Segregation ratios were compared to Mendelian 3:1 ratio using chi-square analysis.

RESULTS AND DISCUSSION

A pGreen II 0229-2ab plasmid modified to have a 35S expression cassette was used for plasmid construction. Plasmids selected with kanamycin resistance were further confirmed by restriction digestion of isolated plasmids. Finally, plasmid sequencing carried out using 35S primers revealed the presence of the correct *CnANT* fragment starting from ATG start codon to TGA stop codon. Transformed plants were screened by spraying the herbicide Harvest which contains the active ingredient gluphosinate ammonium. The PCR products of the twenty six plants survived after herbicide treatment is shown in Fig. 2. Plants were checked for the *CnANT* insert by PCR using 35S primers. Five of them showed a band close to 2000 bp which is expected to produce from the 35S:*CnANT* plasmid (Fig. 2, lane Pl) while other plants only contained the plasmid DNA. These positive plants were grown to maturity at controlled environment and used for the production of transgenic lines (Tg02, Tg05, Tg10, Tg15, and Tg26).

Fig. 2. PCR screening of primary transgenic plants.

Twenty six transgenic plants were screened using primers designed from 35S promoter and terminator. Plants with correct gene insertion showed a band closer to 2000 bp. M: Hyperladder I, 1-26: primary transgenic plants, Pl: 35S:*CnANT* plasmid.

Transgene segregation was analyzed in the seedlings of each line. For each line fifty seeds were sown initially, however some seeds did not germinate on compost. Therefore, each line 17-47 seedlings were finally tested for herbicide survival. All the lines showed the typical 3:1 segregation ratio (Table 2) suggesting that the bar genes had integrated into the *Arabidopsis* as a single segregating locus. After selecting up to third generation (T_3 plants), PCR analysis was performed on genomic DNA to confirm the presence of bar gene and *CnANT* insert (data not shown).

Selected five transgenic lines were given names Tg02, Tg05, Tg10, Tg15 and Tg26. According to the morphological observations, transgenic lines Tg05 and Tg15 were grouped as moderate lines while Tg02, Tg10 and Tg26 were grouped as severe lines. Moderate lines were more similar to wild type plants with mild vegetative and reproductive phenotypes while severe lines exhibited extreme vegetative and floral phenotypes with considerable reduction of fertility. *BBM* is the well known member of ANT sub group which has been reported to induce spontaneous somatic embryos and shoots in seedlings and explants when ectopically expressed. Further, expression of the gene in older tissues (*e.g*. leaves, flowers) have caused pleiotropic morphological alterations (Boutilier *et al.,* 2002). Recently, *AtEMK* which is also known as *AIL5* has shown similar changes when over expressed in *Arabidopsis* (Tsuwamoto *et al.,* 2010). Both *BBM* and *AtEMK* have shown different levels of phenotypic severity as in *CnANT*.

Seed germination was slightly delayed in 35S:*CnANT Arabidopsis* lines compared to wild type plants. Several pleiotropic phenotypes were observed even at seedling stages. These include thickened hypocotyls, elongated cotyledons and anthocyanin accumulation in first whorl of leaves. These characters were more prominent in severe lines than moderate lines. However, no spontaneous embryo production was detected at seedling stage as described for *BnBBM*, *GmBBM* or *AtEMK* (Boutilier *et al.,* 2002; Tsuwamoto *et al.,* 2010). Similarly, in transgenic tobacco Bn*BBM* expression did not induce spontaneous embryogenesis (Srinivasan *et al.,* 2007) but it was induced when a cytokinin pulse was given. Moreover, *EgAP2-1*, oil palm *AIL5-like* gene also was not able to induce spontaneous embryos in transgenic *Arabidopsis* seedlings (Morcillo *et al.,* 2007). These observations suggest that the expression of *BBM* and *AIL5* show fundamental differences in different species even though they activate the same biological process.

Table 1. Segregation analysis of herbicide expression in T1 seeds of transgenic lines.

Transgenic line	Herbicide survival (survived/dead)	Expected segregation ratio (survived/dead)	χ^2 Value[1]	P value
Tg02	27:8	3:1	0.0857	0.70
Tg05	33:12	3:1	0.0667	0.80
Tg10	30:12	3:1	0.2857	0.70
Tg15	30:11	3.1	0.0732	0.80
Tg28	12:5	3:1	0.3269	0.50

[1] χ^2 values are shown for the closest expected Mendelian ratios. These values do not exceed the critical value (3.84) for a 0.05 probability level and thus indicate that the observed frequencies are not significantly different from expected.

Arabidopsis plants harboring the 35S:*CnANT* grew slowly at initial stages producing higher number of rosette leaves. Wild type plants had broad elongated leaves, occasionally with wrinkled margins while severe lines had backward curly leaves with serrated margins (Fig. 3 F). Moderate lines differ from severe lines in terms of leaf serration in which they were more similar to wild type plants. The rosette leaves of the severe transgenic lines at the seed maturity stage were well developed densely arranged and took a bushy appearance (Fig. 3B) compared to moderate and wild type plants.

Ectopic expression of *CnANT* caused significant delay in flowering (Table 3; Fig. 3 A) in which these results are consistent with *BnBBM* overexpressing transgenics as reported by Ouakfaoui *et al* (2010). The number of leaves produced at the flowering time was also significantly increased in 35S:*CnANT* lines as compared to wild type plants. Wild type *Arabidopsis* started flowering after 21.6 ± 0.18 d and the number of leaves at flowering was 8.25 ± 0.41. Tg02 and tg10 lines bolted after 36 ± 1.5 and 37.6 ± 1.7 d when they have produced 22.2 ± 0.36 and 22 ± 0.53 leaves respectively. Tg28 which was grouped as a member of the severe line, slightly deviated from other two counterparts when consider the flowering date. It started flowering after 27.3 ± 0.70 d after producing 11.25 ± 0.37 leaves. In contrast, two moderate lines tg05 and Tg15 flowered slightly later than Tg28 line after 29.25 ± 0.52 and 30.87 ± 0.51 d, respectively. At this age these lines have produced 13 ± 0.19 and 14.12 ± 0.55 leaves, respectively.

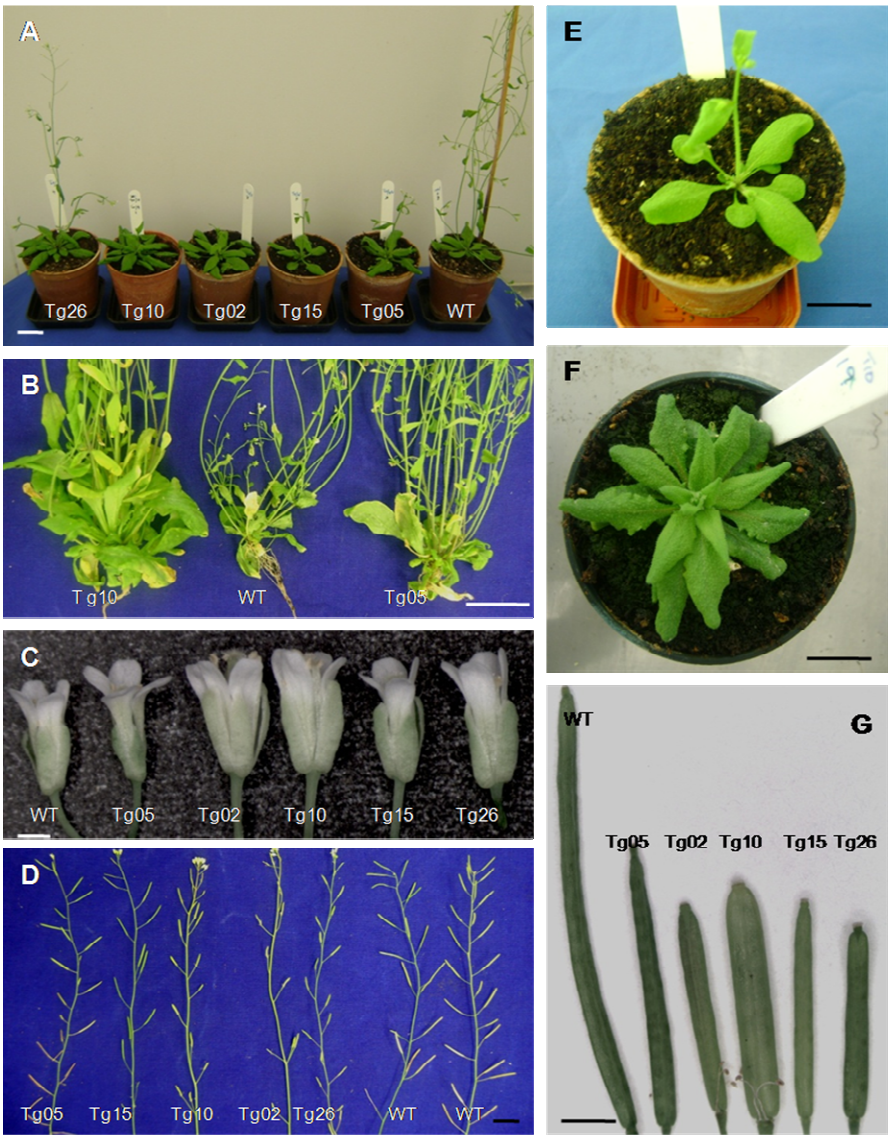

Fig. 3. **Pleiotropic phenotypes caused by ectopic overexpression of *CnANT*. A: 35S:*CnANT* plants showing delayed flowering (after 35 days), B: wild type and 35S:*CnANT* rosettes after 50 days. C: Flowers of transgenic and wild type plants. D: Bolts of five 35S:*CnANT* lines exhibiting the sporadic silique setting. E: wild type *Arabidopsis* Col plant (28 days old). F: Tg10 35S:*CnANT* plant with backward curly leaves (28 days old). WT: Wild type; Transgenic lines: Tg02, Tg05, Tg10, Tg15 and Tg26**

Bars 4 cm in A, 5 cm in B, 2 mm in C, 1.5 cm in D, 2 cm in E & F, 2 mm in G

Table 3. Phenotype statistics[1] of 35S:*CnANT* transgenic plants and wild type plants.

Parameter	Tg02	Tg05	Tg10	Tg15	Tg26	Wild type
Days to flower	36.00±1.4*	29.25±0.5*	37.62±1.6*	30.87±0.5*	27.37±0.7*	21.62±0.19
No. of leaves at flowering	22.25±0.3*	13.00±0.1*	22±0.53*	14.12±0.5*	11.25±0.3*	8.25±0.41
No. of secondary inflorescences	7.00±0.27*	7.00±0.27*	7.62±0.26*	6.75±0.25*	8.00±0.26*	4.75±0.37
Length of sliques (mm)	9.54±0.06*	11.84±0.0*	9.67±0.07*	11.85±0.8*	6.27±0.05*	16.06±0.06
% Production of empty siliques	56.35*	24.27*	53.96*	24.06*	50.65*	0.73

[1]The data are shown as average ± standard error. *, values are significantly difference from wild type. (P>0.001)

During the reproductive phase, 35S:*CnANT* plants exhibited an increased number of secondary inflorescences, decreased silique size and decreased seed set (Table 3). The axils of the rosette leaves produced higher number of secondary inflorescences (more than 6) in 35S:*CnANT* transgenic lines (both severe and moderate) compared to wild type plants (less than 5). However, larger flowers were seen only in severe plant lines (Fig. 3C). *CnANT* appears to be involved in cell proliferation activities in different organs eventually giving rise to larger leaves and flowers than the wild type plants. These results are consistent with the results obtained for *ANT* which generates large flowers and large leaves when ectopically expressed in *Arabidopsis* (Mizukami & Fischer 2000; Nole-Wilson *et al.,* 2005). Further, the results obtained are similar to that of *AIL5* which produces larger flowers in *Arabidopsis* (Nole-Wilson *et al.,* 2005; Tsuwamoto *et al.,* 2010). The fact that *ANT* enhances cell division has been determined by identifying an increase in lateral organ size by increasing cell number rather than increasing the cell size, in gain of function transgenic plants via ectopic expression. On the other hand, the loss of function mutant of *ANT* reduces the organ size by decreasing the cell number (Mizukami & Fischer 2000). Similarly, an increase in floral organ size has been characterized by an increased cell number when *AtMYB118*, another embryogenesis related gene is over expressed (Zhang *et al.,* 2009). Hence, the similar hyperplasia in 35S:*CnANT* transgenic plants may have resulted from enhanced cell division. Despite producing larger leaves in higher number, plants over expressing *CnANT* gave rise to other leaf morphological abnormalities such as serrated margins and backward leaves which have been observed when other AP2/EREBP family members such as *LEAFY PETIOLE* (*LEP*) (Van der Graa *et al.,* 2002), *BBM* (Boutilier *et al.,* 2002), *AtEMK* (Tsuwamoto *et al.,* 2010) and *PLT* (Aida *et al.,* 2004) were over-expressed.

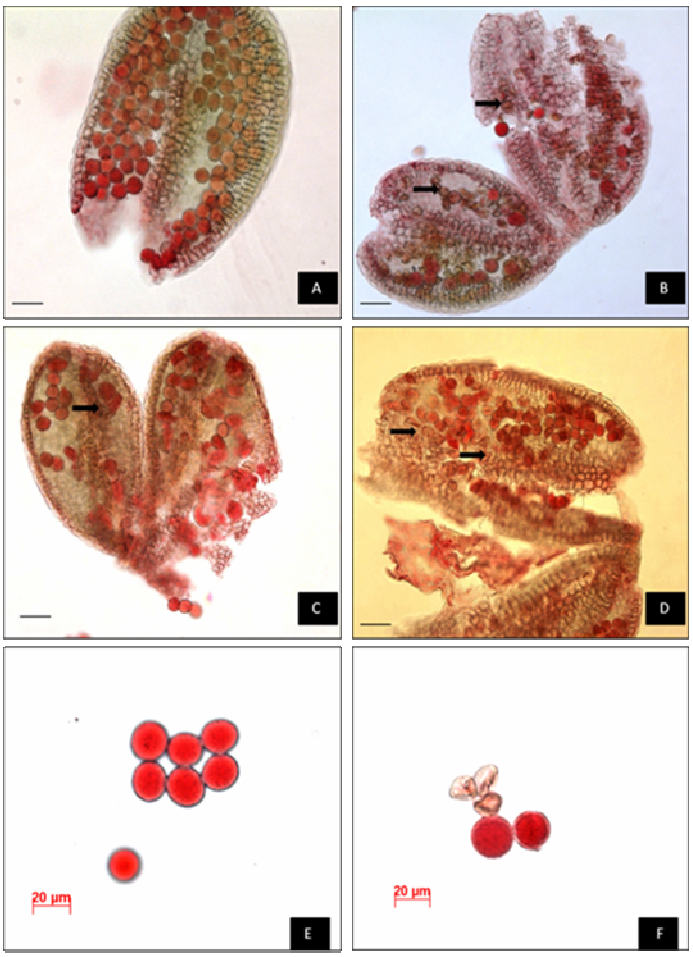

Fig. 4. Comparison of pollen structure of 35S:*CnANT* and wild type plants. Mature pollen grains from wild type (A) and three 35S:*CnANT* lines Tg26 (B), Tg05 (C), Tg10 (D) were stained with acetocarmine. Viability staining of transgenic line pollen showed that the ectopic expression of *CnANT* cause pollen degeneration (F) compared to wild type pollen (E).

Bars 100 µm in A, B,C and D; 20 µm in E and F

Tg02, Tg10 and Tg26 plants showed a significant reduction in silique size compared to wild type counterpart (Fig. 3G and Table 3). The siliques of moderate transgenic lines were slightly longer in length than the severe lines. However, they were always shorter in size compared to the wild type plants. Moreover, the severe 35S:*CnANT* lines showed persistent anthers in siliques indicating delay in senescence of anthers (Fig. 3G). This kind of phenotype has been recorded when soybean *BBM* (*GmBBM1*) is ectopically over expressed in *Arabidopsis*. The inflorescences of transgenic plants harboring 35S:*CnANT* exhibited a sporadic setting of siliques, where some of the siliques fail to set any seeds. Along the inflorescence, these empty siliques (unfertilized carples) were arranged with filled siliques (Fig. 3D). The production of empty siliques was observed to be higher in severe transgenic lines. The percentage production of sterile siliques ranged within 50-56 in these severe lines

(Table 3). Approximately 24 % of siliques were sterile in moderate 35S:*CnANT* lines. This phenomenon appears to represent male sterility in *CnANT* transgenic plants. Male sterility was further evident when pollen grains of transgenic plants were examined under light microscope. All the pollen grains of wild type plants were of round shape and densely stained with acetocarmine (Fig. 4A and E). No aborted pollens were visible. However in 35S:*CnANT* transgenic lines, considerable amount of aborted pollen grains were observed (Fig. 4, B, C, D and F). Since *CnANT* has shown higher expression levels in embryos it was not surprising to see obvious phenotypes in the siliques of 35S:CnANT plants.

CONCLUSIONS

Similar phenotypic effects observed for *CnANT* along with *BBM*, *AIL5*, *ANT* and other *AIL* genes suggest that similar biochemical targets are governed by the proteins encoded by *CnANT* and other AP2 family members. The gain of function phenotype similarities of these genes suggest that the genetic pathways governed by these genes may regulate the same set of target genes when over expressed. Similarity of their expression, thus suggest that *CnANT* gene also has a potential to increase *in vitro* regeneration response as previously characterized *BBM* members of other species.

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