

## CONSTRUCTION OF *GmNAC085* VECTOR FOR FUTURE DEVELOPMENT OF DROUGHT-TOLERANT CROPS

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Received: 02.11.2015

Accepted: 30.6.2016

### SUMMARY

Various members of NAC transcription factor family have been shown to play important roles in regulating plant responses to abiotic stresses, such as drought, cold and salinity. Our previous research on differential expression patterns of twenty three soybean *NAC* genes (*GmNACs*) by realtime quantitative PCR suggested a correlation between inducible expression of *GmNAC085* and the drought tolerance degree in DT51 and MTD720 soybean cultivars, which presented for the drought-tolerant and the drought-sensitive, respectively. Therefore, the gene has been proposed as a potential candidate for engineering in order to produce new varieties with better drought stress tolerance. However, functional studies of *GmNAC085* should be carried out to identify how this transcriptional factor can contribute in the plant stress-responsive pathway. Herein, this paper presented that we have successfully developed a recombinant binary vector carrying full-length cDNA of *GmNAC085* and expression of this gene is placed under the control of constitutive promoter *CaMV 35S*. The generated construct was firstly transformed into *E.coli* for sequencing the target gene and then the identified genuine construct was transformed into *Agrobacterium tumefaciens*. This can be used for plant transformation mediated by *Agrobacterium* in serving for *GmNAC085* - related studies using *in planta* system such as the model plant *Arabidopsis* and also serving for the development of drought-tolerant crops by genetic engineering. Additionally, results from sequence alignment analysis revealed that *GmNAC085s* of DT51 and MTD720 had identical nucleotide sequence, thus supporting our hypothesis that difference in *GmNAC085* gene expression levels, not the variation in *GmNAC085* protein sequence or structure, might cause the difference in plant resistance degree to drought stress in these two soybean varieties.

**Keywords:** Drought-responsive gene, DT51, *GmNAC085*, MTD720, recombinant vector

### INTRODUCTION

Abiotic stresses or non-living adverse factors such as drought, flood, high salinity and extreme temperatures have negative impacts on the growth and productivity of plants. Generally upon the stress exposure, a series of undesired changes in plant morphological, physiological, biochemical and molecular characteristics will occur (Shinozaki, Yamaguchi-Shinozaki, 2007). In agricultural production, abiotic stresses cause significant loss in average yields, including those of major economically important crop plants (Manavalan *et al.*, 2009; Tran, Mochida, 2010). Noticeably, drought is the predominant stress because of its high frequency of appearance, and further being exacerbated by the increase in the world population,

deforestation and global climate change. These challenges have been causing pressure going with the raise in water demand, which might lead to the shortage of water in future for both human life and agriculture activities. Other factors such as level and distribution of rainfall, evaporative degrees and moisture storing capacity of soils also make the severity of drought more difficult to control (Farooq *et al.*, 2009). Drought stress in soil limits crop yield by three main mechanisms, which are reducing utilized efficiency of radiation, decreasing canopy absorption of photosynthetic active radiation, and lowering harvest index (Chaves *et al.*, 2009).

Albeit unlike animals that are able to move for escaping stress, plants also establish a distinct array of strategies to cope with environmental stresses. Upon the stress signal sensed by plants is the

activation of important cell signaling pathways and cellular responses, such as the production of stress-responsive proteins, up-regulation of antioxidants and accumulation of compatible solutes (Chaves *et al.*, 2003). Plants adapt to drought conditions by tightly regulating specific sets of genes in response to drought stress signals, although the drought-tolerant mechanisms in plants have not been completely elucidated. The plant-responsive pathways have been tracked down mainly via molecular studies. Based on such analyses, a number of NACs (NAM- no apical meristem; ATAF- *Arabidopsis* transcription activation factor; CUC- cup-shaped cotyledon) involved in mediation of ABA-dependent and ABA-independent signal transduction and gene expression have been known to participate in drought stress-responsive network of plants. These proteins have been considered as one of the novel classes of plant-specific transcription factors (TFs) functioning in diverse and vital physiological processes during plant development (Meng *et al.*, 2007). A typical NAC protein comprises of highly conserved DNA-binding domain at the *N*-terminus, and diversified transcription regulatory region at the *C*-terminus (Tran *et al.*, 2009). In the last decade, advances in genomic sequencing and bioinformatics have supported the identification of NAC family members in a number of species, such as 117 genes in *Arabidopsis*, 151 in rice (*Oryza sativa*), 152 in tobacco (*Nicotiana tabacum*), and approximately 200 members in soybean (*Glycine max*) (Thao *et al.*, 2013). Many studies showed that regulation of *NAC* expression can lead to the change in plant immunity to stress. Taking study of a rice *NAC* gene as an example, *SNAC1*-overexpressing transgenic plants had higher seed setting rate by 22-34% in the field than the non-transgenic plants under severe drought stress conditions at the reproductive stage (Liu *et al.*, 2014). This result indicated potential applications of other *NAC* genes in genetic engineering for improving drought tolerance in plants.

Regarding *GmNAC* gene family in soybean, a search in Genevestigator database indicated that along with other two genes *GmNAC043* and *101*, *GmNAC085* was highly inducible by various treatments, such as biotic stress, alkaline stress and photoperiod change, as well as showed development stage - related gene expression. Particularly, *GmNAC085* was also found as one of the most prominent *GmNAC* candidates that could play roles in response to not only drought but also other kinds

of abiotic stresses in soybean (Thu *et al.*, 2014a). According to Le *et al.* (2011), *GmNAC085* was elicited as the most dehydration-inducible gene, with 390-fold and 20-fold induction in shoots and roots, respectively. Other studies also revealed that *GmNAC085* was highly induced by drought in both root and shoot tissues of soybean cultivar displaying drought-tolerant phenotype (Thao *et al.*, 2013; Thu *et al.*, 2014b). In addition, encoded protein by *GmNAC085* exhibited 39% identity and 50% similarity to the most extensively characterized rice NAC TF (*SNAC1/ ONAC02*) with positive relation to plant stress tolerance to drought (Thao *et al.*, 2013). These altogether suggested that *GmNAC085* might have potential use in genetic engineering for improvement of plant tolerance to various types of stresses including drought, as thus it is necessary to carry out in-depth functional characterization of this gene in future research.

Being part of a research project in dissecting the function of *GmNAC085* in *planta* in relation to drought response, we report here that a recombinant binary vector for overexpressing *GmNAC085* had been constructed, and already transformed into *Agrobacterium tumefaciens*. These materials can be used for future study in characterizing the role of *GmNAC085* in aiding plants to cope with drought stress. In addition, comparison between DT51 *GmNAC085* sequence and MTD720 *GmNAC085* sequence was also performed. The results indicated that these two cultivars with contrasting phenotype in drought tolerance have no difference in their *GmNAC085* nucleotide sequences.

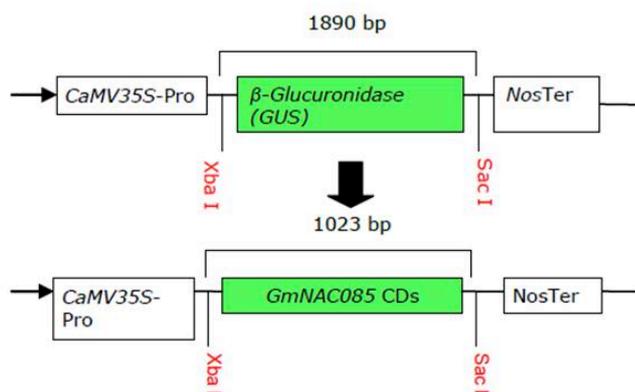
## MATERIALS AND METHODS

### Isolation of *GmNAC085* gene

cDNA templates synthesized from total mRNA that were extracted from the drought-tolerant soybean cultivar DT51 and the drought-sensitive soybean cultivar MTD720 in study of Thao *et al.* (2013) were used to isolate *GmNAC085*. The full length of *GmNAC085* sequence (Glyma12g22880.1) was retrieved from soybean genome database (<http://soybeantfdb.psc.riken.jp/>) and used to design *GmNAC085*-specific primers. The forward (*NAC85F2*: 5'-GGTCTAGAATGGGAGTTCCAGA GAGA-3') and reverse (*NAC85R2*: 5'-GT GAGCTCTCAGTCCCTAAACCCGAA-3') primers also contained restriction sites for *Xba*I and *Sac*I, respectively. The coding sequence of *GmNAC085* was

PCR-amplified by using Phusion High-Fidelity DNA Polymerase kit and GC buffer (New England BioLabs) in 25 µl reactions, each including 1 µl of cDNA template, 200 µM of dNTPs, 0.5 µM each of designed primers and 1 U of Phusion DNA polymerase. The PCR thermal cycling condition was 30 seconds at 98°C,

30 cycles of 98°C for 10 seconds, 57°C for 30 seconds and 72°C for 35 seconds, and followed by a final extension of 10 minutes at 72°C. The PCR products were visualized by gel electrophoresis using 0.8% agarose gel and then purified by GeneJET PCR purification kit (Thermo Scientific).



**Figure 1.** Recombinant vector was constructed with *pBI121* as vector backbone and using restriction enzymes *Xba*I and *Sac*I to replace *GUS* gene by the target gene *GmNAC085*.

### Cloning of *GmNAC085* into binary vector

The amplified *GmNAC085* was cloned into the T-DNA region of binary vector *pBI121* (kindly provided by Dr Nguyen Tuong Van, Institute of Biotechnology, Hanoi, Vietnam), which also contains *NPTII* for selection of the transformed plants by kanamycin. In summary, the  $\beta$ -*Glucuronidase (GUS)* gene on *pBI121* was replaced with *GmNAC085* sequence using *Sac*I and *Xba*I (Thermo Scientific) followed by ligation using T4 DNA ligase (Thermo Scientific) (Fig 1). To increase the ligation efficiency, digested vector was treated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) prior to ligation. The generated construct was transformed into *E. coli* DH5 $\alpha$  competent cells by heat-shock method. Putative transformants growing on LB agar containing kanamycin (50 µg/ml) were confirmed by colony PCR using OneTaq™ Hot Start 2x Master Mix with Standard Buffer (New England BioLabs) and using primers that anneal to the promoter (35SF: 5'-AACCACGTCTTCAAAGCAAGTGGGA-3') and terminator (NosR: 5'-GTATAATTGCGGACTCTAATCATAA-3') of the target gene. The PCR

cycling condition was 94°C for 3 minutes, 30 cycles of 94°C for 15 seconds, 48°C for 1 minute and 68°C for 2 minutes and 20 seconds following by a final extension of 5 minutes at 68°C. The size of PCR amplicons was determined by DNA gel electrophoresis.

### DNA sequencing

The recombinant plasmid was multiplied and extracted from the transformed *E. coli* by using GeneJET Plasmid Miniprep Kit (Thermo Scientific). The vectors were sent to 1<sup>st</sup> BASE DNA Sequencing Services (Malaysia) for sequencing *GmNAC085* using NAC85F2, NAC85R2 and 35SF primers. The obtained *GmNAC085* sequences of DT51 and MTD720 were aligned with the reference *GmNAC085* sequence from soybean cultivar Williams 82 (W82) (ID Glyma12g22880.1). Multiple sequence alignment was performed using Clustal Omega multiple sequence alignment tool.

### Transformation of recombinant vector into *Agrobacterium tumefaciens*

The extracted recombinant vector was transformed

into *A. tumefaciens* EHA101 competent cells by freeze-thaw method (Weigel, Glazebrook, 2005) with modifications. The competent cells were prepared using protocol mentioned in Yong *et al.*, (2006). Transformed bacteria were identified using LB-agar containing 50 µg/ml rifampicin and 50 µg/ml kanamycin after two-day incubation at 28°C. The confirmation of true transformants was also conducted by colony PCR.

## RESULTS AND DISCUSSION

### Successful isolation of *GmNAC085*

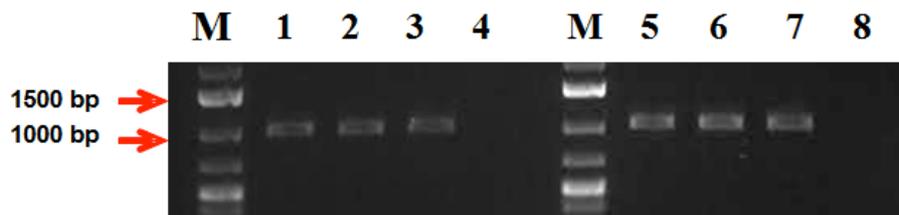
Among fourteen soybean cultivars examined in the research of Thu *et al.*, (2014b), DT51 displayed the strongest drought-tolerant phenotype while MTD720 showed the highest drought-sensitive phenotype. Detailed analyses on expression of a number of *NAC* genes in these two cultivars under normal and drought stress conditions revealed a similar up-regulation tendency of *GmNAC085*, although with higher expression levels in DT51 roots and shoots than in MTD720 root and shoot counterparts (Thao *et al.*, 2013; Thu *et al.*, 2014a). Therefore, it is interesting to find out whether *GmNAC085* sequences in DT51 and MTD720 are the same or not.

In order to find out this answer, we decided to pull out full coding sequence of *GmNAC085* genes using cDNA templates from these two soybean varieties, which had been synthesized from our previous study (Thao *et al.*, 2013). The results obtained by gel

electrophoresis indicated that only one product was made in each PCR reaction. In addition, the amplified PCR products from both templates had the same size and also agreed with the size of the reference *GmNAC085* from W82 soybean cultivar (Fig 2). The size of *GmNAC085* cDNA was 1023 base pairs (bp) while the total size of PCR products including the sequence of primers was 1035 bases.

### Successful cloning of the *GmNAC085* genes into binary vector and successful transformation of the recombinant vector into *E. coli*

After digesting amplified *GmNAC085* PCR products and binary vector *pBII121* using the same restriction enzymes *XbaI* and *SacI*, the cut samples were examined on electrophoretic gel. According to Figure 3A, the sizes of bands representing for digested PCR products that had been amplified from cDNA template from DT51 (Lane 1) and MTD720 (Lane 2) cultivars were pretty similar to uncut sequences (around 1035 bp). Meanwhile, after digesting the vector (before ligation) with 14758 bp in size by *SacI* and *XbaI*, there were two bands observed in the lanes loading the digested vector and also matched with the expected cut sizes, which are 1900 bp (containing the *GUS* gene) and 12858 bp (Fig 1, Fig 3B, Lane 3). These results implied that complete digestion was achieved and ready for purification following by ligation reaction to join the target gene *GmNAC085* with the 12858-bp fragment of the vector.



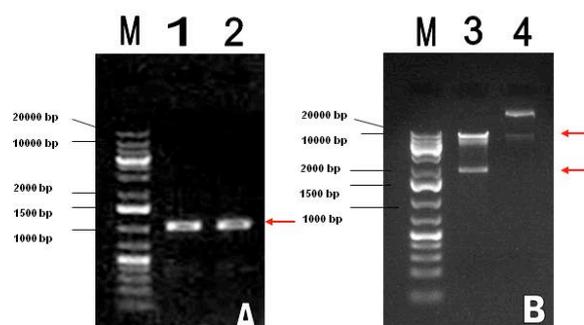
**Figure 2.** Band sizes of PCR products with cDNA templates from DT51 (A) and MTD720 (B) soybean cultivars using primer pair specifically designed for *GmNAC085*. Lane M: DNA 1kb ladder (Fermentas); Lanes 1-3: Amplified PCR products from template of DT51; lanes 4 and 8: Negative controls; Lanes 5-7: Amplified PCR products from template of MTD720. The predicted size for successful amplified *GmNAC085* including primers is 1035 bp.

By applying heat-shock method, ligated vector-inserts were introduced into *E. coli* competent cells. After overnight incubation, *E. coli* colonies appeared on selective LB agar plates containing kanamycin (50 µg/ml). To ensure the putatively transformed *E. coli* colonies did possess the expected

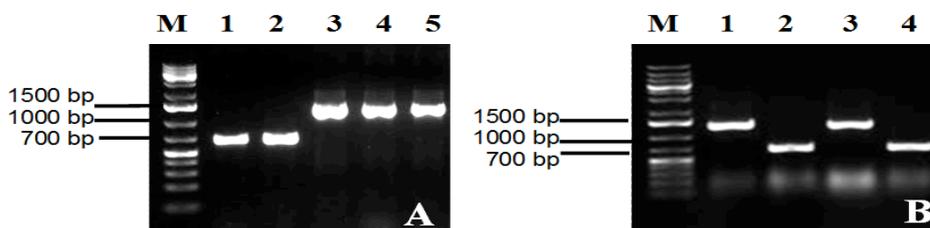
recombinant vector (*pBII121-GmNAC085*), colony PCR was performed for a number of randomly chosen colonies using 35SF and NosR primers for checking the presence of inserted gene. Obtained results from gel electrophoresis showed that the PCR products had two different sizes (Fig 4). One size

was approximately 700 bp, and the other was in the range of 1000 bp and 1500 bp in length. By using the 35SF and NosR primers, the correct PCR product should have the size of 1347 bp. The explanation for obtaining the unexpected colony PCR result (700 bp) could be due to the primers NAC85F2/NAC85R2 used for PCR reaction to isolate *GmNAC085* was not really specific for the target gene and thus they possibly annealed to another DNA template and generated a non-specific PCR product. This non-

target product has been joined with the vector by enzymatic restriction and ligation reactions. The amount of this sub-product probably was not as high as the main PCR product and thus it could not be seen on the electrophoretic gel in previous steps (Fig 2 and Fig 3). Based on the results, therefore, colonies 3, 4, 5 (DT51 variety) (Fig 4A), and colonies 1 and 3 (MTD720 variety) (Fig 4B) showing the right bands so these were used to prepare sample for sequencing in the next step.



**Figure 3.** Gel electrophoretic results of digested PCR products and binary vector using *Xba*I and *Sac*I before ligation. Lane M: DNA 1 kb ladder (Fermentas); Lane 1: digested *GmNAC085* PCR product using DT51 template; Lane 2: digested *GmNAC085* PCR product using MTD720 template; Lane 3: digested *pBI121* vector; Lane 4: uncut *pBI121* vector.



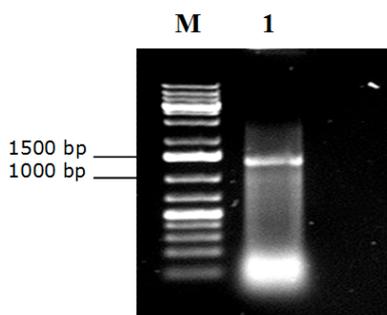
**Figure 4.** Gel electrophoretic results for colony PCR of transformed *E. coli* with *GmNAC085* amplified from DT51 cDNA (A) and from MTD720 (B). Lane M: 1kb DNA ladder (Fermentas); Lanes 1-5: putatively transformed colonies numbered 1 to 5 used for colony PCR. The primers used for the PCR were 35SF and NosR.

#### DNA sequencing revealed that DT51 and MTD720 had the same *GmNAC085* sequence as of W82

A problem can be seen when sequencing is the inaccurate identification of nucleotides due to gap (non-identified nucleotide) or variation within regions of the first or final 100 bases counted from the position that the primers binding to the template. Therefore, to ensure a full, confirmed sequence of *GmNAC085* can be retrieved, we used three different primers binding at different positions either near or within the target gene. By doing so, *GmNAC085* sequences of DT51 and MTD720, respectively, were successfully obtained with 1023 bp for the coding length for both varieties. When performing alignment

against the *GmNAC085* sequence of the reference W82 cultivar, whose genome sequence has been available, the results showed that *GmNAC085* in DT51 and W82 had the same sequence (Fig 5). The sequence alignment for this gene between MTD720 and W82 also displayed the same result (data not shown). Combine analyses from this study and from previous studies conducted by our group, therefore, it is indicated that the correlation of *GmNAC085* and drought tolerance ability is due to the level of *GmNAC085* transcripts and corresponding proteins which have been synthesized, not because different cultivars (DT51 versus MTD720 in our study case) had *GmNAC085* with difference in structure (Thao *et al.*, 2013; Thu *et al.*, 2014a; Thu *et al.*, 2014b).





**Figure 6.** Gel electrophoretic results for colony PCR of transformed *Agrobacterium* with *GmNAC085* amplified from DT51 cDNA. Lane M: 1kb DNA ladder (Fermentas); Lane 1: putatively transformed colony growing on LB-agar containing rifampicin (50µg/ml) and kanamycin (50µg/ml). The primers used for the PCR were 35SF and NosR.

#### Successful transformation of recombinant vector into *Agrobacterium tumefaciens*

The verified recombinant vector with correct *GmNAC085* was transformed into *A. tumefaciens*. Similar to *E.coli*, the transformed *Agrobacterium* was identified by using antibiotics and colony PCR. As shown by Figure 6 for the result of colony PCR using primers 35SF and NosR, only one band was visualized on the gel with the expected size for the amplified product (1347 bp). This implies that our transformation was achieved.

#### CONCLUSION

In this paper, we reported that we have successfully constructed recombinant vector carrying *GmNAC085* under control of a constitutive promoter and transformed into *Agrobacterium* for future characterization of its role in response to abiotic stresses *in planta*, especially under drought condition. Another finding obtained from this study is that the correlation hypothesis of *GmNAC085* and drought tolerance by DT51 and MTD720 as proposed by previous research is solely due to the differences in *GmNAC085* expression, not the gene sequence.

**Acknowledgements:** This research was funded by The International University, VNU-HCM under grant numbers T2014-08-BT and SV2014-BT-14.

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## THIẾT KẾ VECTOR TÁI TỔ HỢP MANG GEN *GmNAC085* NHẪM PHÁT TRIỂN GIỐNG CÂY TRỒNG CHỊU HẠN

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### TÓM TẮT

Một số thành viên thuộc họ nhân tố phiên mã NAC có vai trò quan trọng trong điều hòa đáp ứng của thực vật với các stress phi sinh học như stress hạn, stress lạnh và stress mặn. Các nghiên cứu trước đây của chúng tôi về sự biểu hiện của hai mươi ba gen *NAC* (*GmNACs*) ở hai giống đậu tương, một chịu hạn tốt (DT51) và một chịu hạn kém (MTD720), bằng phương pháp định lượng PCR đã đề xuất mối tương quan giữa biểu hiện cảm ứng gen *GmNAC085* với mức độ chịu hạn của cây. Do đó, *GmNAC085* được đề xuất là một ứng viên tiềm năng phục vụ cho việc tạo ra các giống cây mới có khả năng chịu hạn tốt hơn bằng kỹ thuật di truyền. Tuy nhiên, chức năng cụ thể của *GmNAC085* trước hết cần được tìm hiểu để có thể xác định vai trò điều hòa của nhân tố phiên mã này trong con đường đáp ứng với stress ở thực vật. Trong nghiên cứu này, chúng tôi đã tạo được vector tái tổ hợp mang cDNA của gen *GmNAC085* và sử dụng promoter *CaMV 35S* để điều khiển sự biểu hiện của gen này. Vector tái tổ hợp được biến nạp vào *E.coli* để giải trình tự gen phân lập được trước khi vector mang gen đúng trình tự được chuyển vào *Agrobacterium tumefaciens*. Dòng khuẩn mang vector này có thể dùng để chuyển gen đích vào cây bằng *Agrobacterium* nhằm phục vụ cho các nghiên cứu liên quan đến *GmNAC085* trên thực vật như cây mô hình *Arabidopsis* cũng như để tạo ra các giống cây trồng chịu hạn bằng công nghệ gen. Thêm vào đó, kết quả so sánh trình tự gen *GmNAC085* của DT51 và MTD720 cho thấy không có sự khác biệt về trình tự nucleotide giữa hai giống đậu tương được nghiên cứu, do đó củng cố giả thuyết sự khác nhau về khả năng chịu hạn ở hai giống đậu này có thể là do sự khác nhau về mức độ biểu hiện gen *GmNAC085*.

**Từ khóa:** DT51, gen đáp ứng stress hạn, *GmNAC085*, MTD720, vector tái tổ hợp