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Construction of transgenic arabidopsis expressing *crooked neck* gene from rice

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Abstract

Rice is the most important agricultural product of Thailand. However, its productivity is greatly limited by salt stress in some areas. To encounter with this problem, genome wide association study was conducted to predict the genes involving in salt tolerance in rice. *LOC_Os05g22260* (*Crooked neck gene*, *OsCRN*), the uncharacterized gene was one of the causative genes predicted by this method. The transgenic *Arabidopsis thaliana* expressing *OsCRN* was constructed to facilitate the study of *OsCRN* function. *OsCRN* was cloned into pCAMBIA1301 by partial digestion to avoid the digestion at the middle of structural gene, before inserted into the expression vector, to generate pCAMBIA1301_*OsCRN*. After validation of *OsCRN* sequencing and open reading frame, pCAMBIA1301_*OsCRN* was transferred into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw transformation and used for *Agrobacterium* mediated transformation into *Arabidopsis thaliana* by floral dip method. The transgenic lines were selected and propagated to T_2 generation, and segregation ratio was determined. These transgenic lines will be used to determine the function of the *OsCRN* in the future.

Introduction

Rice is the food that feed billions of people around the world and the important export product of Thailand. However, rice production is greatly limited by abiotic stress, especially salt stress[1]. When plants expose to high salinity, excessive salt reduces water uptake of the roots, causing osmotic stress and followed by ion toxicity, which has many physiological effects on plants, including metabolic disturbance, ion imbalance, and increase in radicals[2].

To predict the gene that involves in salt stress tolerance, which is a quantitative trait, in rice, genome wide association study (GWAS) was performed. This approach is based on the variation of single nucleotide polymorphisms (SNPs) in the populations. According to Chadchawan et al.[3], GWAS had predicted many salt responsive genes in rice by using Thai local rice cultivars. *LOC_Os05g22260* (*Crooked-neck gene*, *OsCRN*), which is uncharacterized rice gene, was predicted as a salt tolerant causative gene. The involvement of *OsCRN* in salt stress response was validated in Arabidopsis T-DNA insertion line possessing the T-DNA insertion in *At5g41770*, the homologous gene of *OsCRN*.

CRN was annotated according to RNA binding property from tetratricopeptide repeat (TPR)[4]. The function of CRN has been characterized in fruit fly and human. In fruit fly, CRN is a component of spliceosome, promoting alternative splicing and function in embryo development[5]. In human, it is component of spliceosome and implicated in splicing process[4]. On the contrary, the function of this gene is unknown in plants.

The concept of alternative splicing as the stress response regulation has been emerging. This process acts as important modulator of gene expression that greatly shapes the salt stress

response, especially abscisic acid (ABA) response[6], which is a plant stress hormone. Therefore, further study on the function of *OsCRN* which tend to be a part of alternative splicing process may greatly benefit the rice breeding program.

However, the construction of transgenic rice is time consuming process. Callus induction and regeneration is required[7]. The Alternative method like floral dipping in *Arabidopsis thaliana*, which does not require labor intensive processes such as tissue culture and vacuum infiltration, can reduce the cost and time to construct transgenic plant[8]. Hence, this study reports the construction of the transgenic Arabidopsis by using floral dipping method to express *OsCRN* in two genetic background including wild type and the T-DNA insertion mutant line to facilitate the study of *OsCRN* function. These transgenic lines will be used to determine the function of the selected salt responsive gene in the future.

Methodology

Construction of expression vector for rice gene overexpression in Arabidopsis

The plasmid clone contains full length cDNA of *OsCRN* was ordered from NIAS DNA Bank (http://www.dna.affrc.go.jp/database/) and used as the template for amplification with Q_5 polymerase (New England Biolabs, USA) by using forward primer containing *Nco*Irestriction site (TTACCATGGCGCTCATCACCCCAGC) and reverse primer containing *BstE*II restriction site (CAGCAAGGTCACCAGCTCAGACATCATCAG) to obtain the CDS of *OsCRN*. The PCR reaction condition was set up according to the manufacturer protocols (New England Biolabs, USA) by using 5 minutes at 95^{o}C followed by 35 cycles of 1 minute at 95^{o}C, 30 seconds at 66^{o}C, and 70 seconds at 72^{o}C prior to 5 minutes of final extension at 72^{o}C. The PCR product was then purified by TIANGEN Universal DNA Purification Kit (Tiangen, China).

The binary vector, pCAMBIA1301 (Figure 1), was used as the backbone plasmid for expression vector construction. pCAMBIA1301 was digested with *Nco*I and *Bst*EII to remove the CDS of *GUS* under control of CaMV35S promoter and NOS terminator, while the *OsCRN* fragment was partially digested with the same restriction enzymes by incubating at 37^{o}C for 5 minutes instead of 1 hour to avoid the digestion in the middle of *OsCRN* structural gene. The expected digestion product was then purified by TIANGEN Universal DNA Purification kit before ligation by T4 ligase (New England Biolab, USA) using 1: 7 vector: insert molar ratio to obtain the expression vector (pCAMBIA1301_*OsCRN*).

Bacterial Transformation

pCAMBIA1301_*OsCRN* was transferred to *Escherichia coli* DH5α by the heat shock transformation[9]. In brief, 100 ng of DNA was added to 50 µl of competent *E. coli*. and incubated at 42^{o}C for 1.5 minutes followed by keeping on-ice for 3 minutes. After that, LB was added to the transformed *E. coli*, and incubated in shaker (250 rpm) at 37^{0}C for 1 hour. The transformed *E. coli* was selected on LB plate, containing 50 µg/ml kanamycin.

The single colonies were picked and resuspended in 10 µl sterilized distilled water to be used as a template for colony PCR with *Taq* polymerase. The PCR reaction was set up as instructed according to the manufacturer protocol (Thermo-Fisher, USA) using 5 minutes at 95^{o}C followed by 35 cycles of 1 minute at 95^{o}C, 30 seconds at 66^{o}C, and 140 seconds at 72^{o}C prior to 7 minutes of final extension at 72^{o}C. The positive colonies that gave the PCR product at the expected size (2.2 kb) was inoculated in LB broth containing 50 µg/ml kanamycin and incubated at 37^{o}C for 18 hours. Next, the *E. coli* cell suspension was checked for the present of *OsCRN* again with the colony PCR in the same condition as mentioned above. The true positive sample was used for plasmid extraction by GeneAid Presto^{TM} Mini Plasmid Kit (GeneAid, USA). The plasmid was submitted for Sanger sequencing (Bioneer, Korea) and the sequences of *OsCRN* fragments were confirmed by pairwise alignment (https://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html) with the sequence of *OsCRN* in the rice genome database (http://rice.plantbiology.msu.edu).

Next, pCAMBIA1301_*OsCRN* was transferred to *Agrobacterium tumefaciens* by freeze-thaw transformation according to Hofgen and Willmitzer[10]. Five hundred nanograms of plasmid was added to 50 µl competent cell, and incubated for 5 minutes on ice, then, 5 minutes in liquid nitrogen, and 5 minutes at 37ºC. After adding 1 ml of LB, the culture was incubated in 250 rpm shaker at 28ºC for 2 hours before plating in LB agar supplemented with 40 µg/ml gentamycin, 20/ml µg rifampicin, and 50 µg/ml kanamycin. The positive clones were checked for the existence of the plasmids by colony PCR as described above.

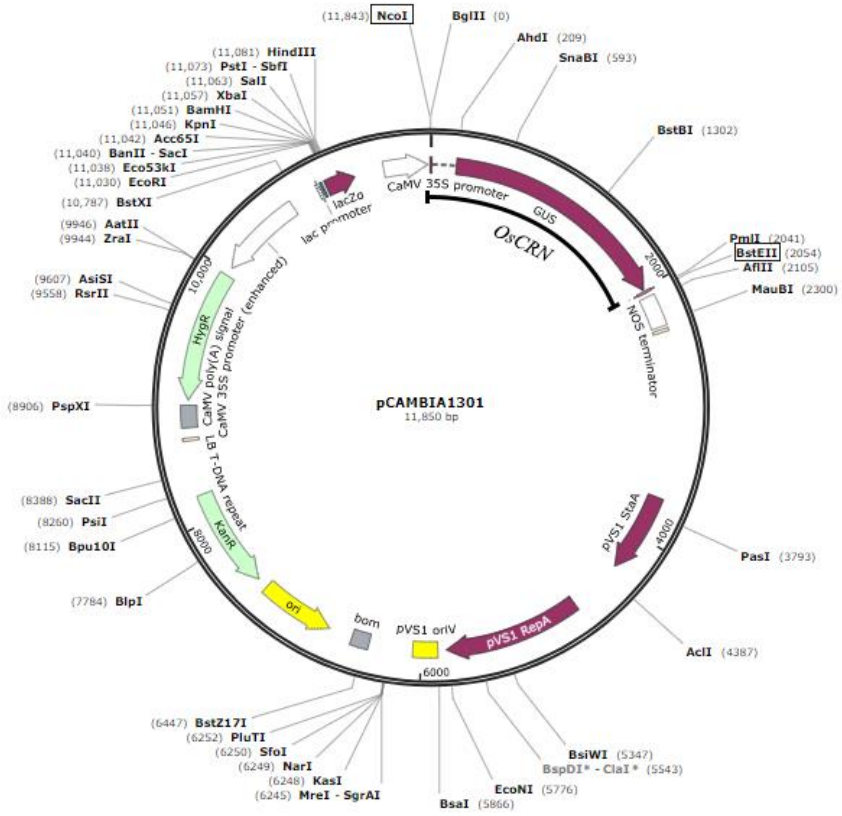

Figure 1. pCAMBIA1301 with position of *OsCRN* cDNA insertion

Construction of transgenic Arabidopsis expressing OsCRN

The plasmid was transformed to *Arabidopsis thaliana* ecotype Columbia-0 by floral dipping method[8]. *A. tumefaciens* culture carrying pCAMBIA1301_*OsCRN* was plated on LB agar supplemented with 40 µg/ml gentamycin, 20 µg/ml rifampicin, and 50 µg/ml kanamycin and incubated at 28ºC for 4 days. Then, *A. tumefaciens* was resuspended in LB medium containing 5% sucrose and 0.05% Silwet-L77 to reach OD_{600} around 1.2. The Arabidopsis bearing closed flowers was dipped in transformation medium for 5 seconds and gently tab during the dipping. After keeping in dark and moist condition overnight, plants were grown normally under 16/8 light-dark cycle at 100-120 µmol photon $m^{-2}s^{-1}$ light intensity at 22ºC.

The transgenic lines were selected according to Harrison et al.[11] The seeds of T_0 (T_1 seeds) were plated on 0.5X Murashige and Skoog (MS) agar containing 20 µg/ml *Hygromycin*-B and stratified at 4ºC under darkness for 48 hours. After that, the plants were exposed to the light for 16 hours and kept in the darkness for 4 days. The plants showing hypocotyl elongation were then selected and transferred to grow under 16/8 light/dark condition for a couple days before transferred to grow on soil. T_2 seeds of each line was germinated on hygromycin plates. The segregation ratio of antibiotic resistant: antibiotic sensitive seedlings was determined and tested by Chi-square at 95 % significant level (p-value = 0.05) in Microsoft Excel.

Genomic DNA of the selected T_1 lines was extracted by Della Porta protocol[12] and PCR with two set of primers: forward (ATCGGCGAGTACTTCTACACAG) and reverse (CTGAACTCACCGCGACGTC) for detecting hygromycin resistant gene (*HygR*) and the same set of primers used for *OsCRN* amplification.

Results and Discussion

Once the *Nco*I and *Bst*EII restriction sites were introduced to *OsCRN*,the partial digestion was performed to avoid digestion at the middle of structural gene of *OsCRN*. The lower fragment (1.2 kb) resulted from the digestion of *Bst*EII, while the upper fragment (2.2kb) was undigested piece mixed with the digested product with the expected digestion at 5' and 3'end (Figure 2).

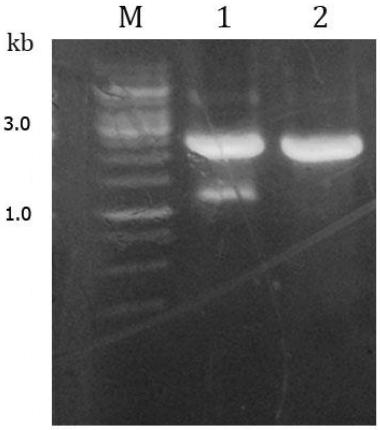

Figure 2. Product of *OsCRN* partially digested by *Nco*I and *BstE*II (lane 1) including the PCR product control (lane 2)

Next, the upper fragment was purified and ligated with pCAMBIA1301 which was cut by *Nco*I and *Bst*EII to remove *GUS* reporter gene. After ligation, the recombinant plasmid was transformed to competent *E. coli*. The ligation reaction from upper fragment resulted in low number of colonies (Figure 3). This might due to the low number of appropriate digested product.

The single colonies were check the present of the insert by colony PCR and restriction digest analysis. The colony that did not show the band might be resulted from the self-ligation of the plasmid. The positive colony was then submitted for Sanger sequencing and the clone with the correct sequence was transferred into *A. tumefaciens*. The success in pCAMBIA1301_*OsCRN* showed that partial digestion can be used as effective protocol for avoid the digestion within the structural gene. Despite alternative method like Gibson Assembly has higher efficiency[13], the partial digestion requires less budget and time.

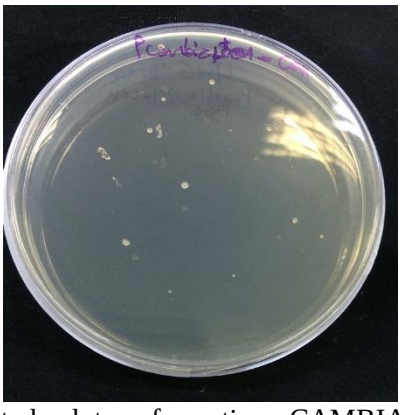

Figure 3. Heat-shock transformation pCAMBIA1301_*OsCRN*

Once the pCAMBIA1301_*OsCRN* was transferred into Arabidopsis by floral dipping, the progeny of transformed plants (T_1) was screened by 20 µg/ml *Hygromycin*-B. The plant contained *HygR* would show hypocotyl elongation (Figure 4A). The present of *OsCRN* and *HygR* were then checked again by PCR (Figure 5). Only some plants possess both *HygR* (Figure 5A) and *OsCRN* (Figure 5B) genes. The absent of *OsCRN* band was due to the incomplete gene transfer which frequently occurred in the transformation with large T-DNA insertion[14]. Based on our screening, 15 positive hygromycin resistant plants by phenotype, showed 1 line, lacking the T-DNA insert and 6 lines containing both *HygR* and *OsCRN* genes (Figure 5). This yielded 40% successful rate of large fragment transformation (≈6 kb), after screening of hygromycin resistant phenotype. By this method, at least 7 lines of the wild type Arabidopsis background and 6 lines of mutant background were generated.

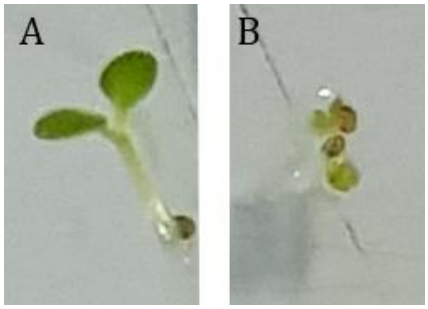

Figure 4. *Hygromycin* selection of transgenic Arabidopsis showing Arabidopsis with *HygR* gene (A) and without *HygR* gene (B)

The progeny of T_1 plants were screened again to determine the segregation ratio of transgenic Arabidopsis. 3: 1 ratio which is the ratio representing the single insertion of transgenes was expected. However, the result (Table 1) showed that most of the insertion was not resulted in 3: 1 ratio and tend to show higher number of ratio which implied to multiple insertion of T-DNA[15].

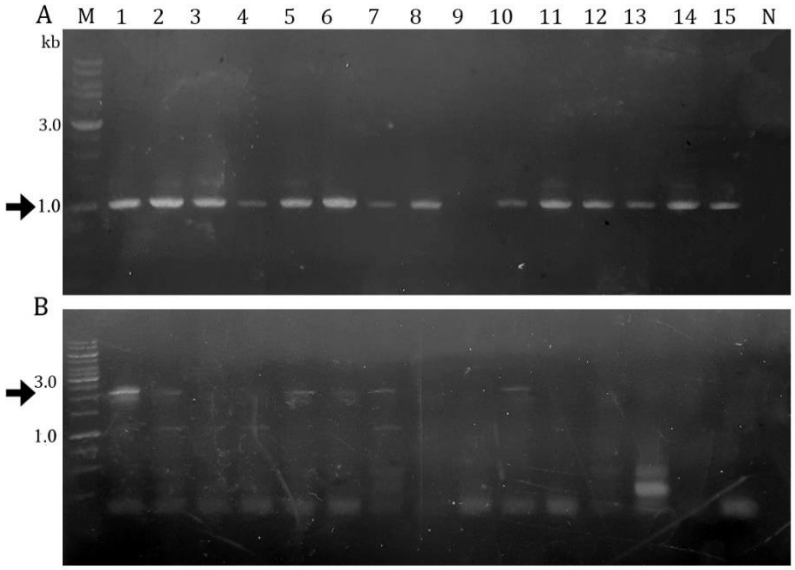

Figure 5. Screening of transgenic Arabidopsis. Fifteen plants (lane 1-15) were screened by *HygR* gene (A) and *OsCRN* gene (B) detection. The negative controls were shown in lane N.

Despite of the multiple insertion of T-DNA, these transgenic Arabidopsis lines can still be a convenient model organism for plant biologist. Apart from comparing between the rice

gene expressing line and wild type Arabidopsis, this approach of study allows the researcher to compare the knock-out mutant lines with rice gene expressing line to characterize the function of plant genes by various methods ranged from simple phenotypic analysis to transcriptome analysis[16]. Moreover, the transgenic Arabidopsis can be crossed to transfer the transgene to another genetic background which is useful for further study in plant molecular biology.

Table 1. Segregation ratio of transgenic Arabidopsis with mutant background

Transgenic lines	Segregation ratio
Revertant A	9:1
Revertant B	14:1
Revertant C	4:1

Conclusion

This research accomplishes to produce transgenic Arabidopsis containing *OsCRN* gene using floral dipping method. *OsCRN* was cloned into pCAMBIA1301 by partial digestion and ligation resulted in pCAMBIA1301_*OsCRN*. The pCAMBIA1301_*OsCRN* was then transferred to *Agrobacterium tumefaciens* and used in floral dipping. At least 7 lines of the wild type background and 6 lines in mutant background were generated. These transgenic lines will allow the researchers to characterize the *OsCRN* gene function in the next step.

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