ECTOPIC EXPRESSION OF OPKN1 GENE IN N. benthamiana
ALTER FLOWER SIZE AND DELAY FLOWERING TIME

MOHD WAZNUL ADLY, M.Z.1,2, ZURAIDA, A.B.2, WEE, C.Y.2, ALIZAH, Z.2, NORLIZA, A.B.2
and CHE RADZIAH, C.M.Z.1*

1School of Biosciences & Biotechnology, Faculty of Science & Technology,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
2Molecular Biology & Genetic Engineering Programme, Biotechnology Research Centre,
Malaysian Agriculture, Research & Development Institute,
43400 Serdang, Selangor, Malaysia
*E-mail: cradziah@ukm.edu.my

ABSTRACT

Oil Palm Knotted-like 1 (OPKN1) gene is a type of homebox gene that was previously isolated from cDNA library of oil palm’s suspension cell culture. Based on semi-quantitative RT-PCR, northern blotting and in situ hybridization analysis, the OPKN1 gene is active in flower meristem suggesting its involvement in flowering phase. Semi-quantitative RT-PCR analysis indicated that OPKN1 gene is active in both male and female flowers and also in abnormal flower suggesting its role in flower morphogenesis process. To further validate the function of this gene, the overexpression transformation vector of OPKN1 gene driven by CaMV 35S constitutive promoter was constructed. The constructed vector was transformed into plant model systems Nicotiana benthamiana via Agrobacterium tumefaciens. Four transgenics lines of N. benthamiana were successfully obtained and analysis of quantitative Real-Time PCR shows that OPKN1 gene was highly overexpressed in all transgenic lines. Overexpression of OPKN1 gene in N. benthamiana transformant has caused reduction in N. benthamiana’s flower size and flowering time was also observed to be delayed. Based on this finding, it was suggested that OPKN1 may be involved in flower development and morphogenesis process.

Key words: Knotted gene, oil palm, OPKN1, gene expression, N. benthamiana

INTRODUCTION

Knotted-like homebox (KNOX) gene is a gene which is classified in big superfamily of Three Amino Loop Extention (TALE). This gene contains conserved homedomain and ELK and also has a conserved KNOX motif known as a MEINOX that functions in protein-protein interaction. The KNOX gene also contains helix motif at ELK domain which is also reported to involve in protein-protein interaction. The TALE structure presents in plant KNOX gene was also found in animal and fungi suggesting it derivation from the same ancestor (Mukherjee et al., 2009). In plants, KNOX is divided into two classes which are class 1 and class 2 depending on the similarity of residue on homedomain and also intron position (Chan et al., 1998). The KNOX gene also was grouped based on gene expression pattern and also the similarity of gene sequence (Kerstetter et al., 1994). Generally, class 1 KNOX gene is mainly expressed at apical meristems and at embryo stage. Previously, it was reported that most of class 1 KNOX was expressed in plant meristem but not at the lateral organ and it is involved in maintenance of plant meristem, leaf development, senescence process, hormone pathways and also involve in flowering structure. In other hand, class 2 KNOX gene show wider range of gene expression pattern in plant (Smith et al., 1992).

Research on Zea mays Knotted-like 1 (ZMKn1) gene show that it is not highly expressed during maize leaf development but it is active at the apical part of maize flower and vegetative. Research conducted on ZMKn1 gene suggested that plant homeobox has important role in cell fate determination. Mutation of ZMKn1 in maize has caused shootless phenotype during cotyledon development (Vollbrecht et al., 2000). Another example of class 1 KNOX gene is SHOOTMERISTEMLESS (STM) gene in Arabidopsis. STM gene have a close relationship with ZmKn1 gene based on the similarity of protein sequence and gene expression pattern which was found expressed at whole part of Arabidopsis meristem (Vollbrecht...
et al., 2000). Long et al. (1996) also reported that Arabidopsis STM gene is a type of class 1 KNOX gene which is essential for shoot apical meristem formation during embryogenesis.

Oil Palm Knotted Homebox or OPKN1 gene is a type of homeobox gene isolated from cDNA library of oil palm suspension cell culture. This 1310 bp gene was found to have a lot of similarity with class 1 protein of knotted-like 1. Based on BLASTX analysis, homeodomain sequence of OPKN1 gene has close similarity with maize knotted-like 1 sequence suggesting it classified under knotted-like 1 group. Che Radziah (2005) have reported that OPKN1 gene was highly expressed in oil palm vegetative and flower meristem suggesting it function in vegetative and reproductive phase for oil palm growth. In addition, the OPKN1 gene was also expressed in oil palm’s embryogenic culture which is indicate that it involvement in development of oil palm’s embryogenesis. However, distribution of OPKN1 activity in oil palm vegetative meristem is differing from others species based on the specific localization of OPKN1 expression in vegetative meristem. Other difference of OPKN1 gene is the expression was highly detected during leaf primordial development. This expression pattern indicated that the involvement of OPKN1 in leaf development especially during changes in leaf morphology from lanceolat to bifurcate and lastly to pinnate in oil palm frond. Overall, OPKN1 do not have specific activity in oil palm part since it was expressed in many phase of oil palm’s development. Since OPKN1 poses a few different pattern of activity, further research is needed to reveal the OPKN1 gene function in more details. Therefore, further study of OPKN1 gene using plant model systems in needed to have more understanding on OPKN1 gene especially in oil palm development.

MATERIALS & METHODS

Construction of OPKN1 expression vector

OPKN1 gene was cloned into pMIR104a vector flanked by CaMV 35S promoter and NOS terminator. The OPKN1 expression cassette was digested and then ligated into pCAMBIA 1305.2 vector. This vector known as pExOPKN1 vector was transformed into Agrobacterium tumefaciens strain LBA 4404 and was further used for Agrobacterium-mediated transformation of OPKN1 gene into N. benthamiana.

Agrobacterium-mediated transformation of OPKN1 gene into N. benthamiana

Transformation of OPKN1 gene into N. benthamiana via Agrobacterium was based on method reported by Hanania et al. (2009). N. benthamiana leaf disks infected with A. tumefaciens carrying pExOPKN1 plasmid were cultured in MS media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. The media was also contains 250 mg/L carbenicilin to eliminate Agrobacterium growth and 40 mg/L hygromycin for selection of transformant N. benthamiana cells. The infected N. benthamiana leaf disks were cultured for 3 weeks and were sub-cultured every 3 weeks. Any plantlet generated from leaf disk was transferred to flask jar containing MSO with 40 mg/L Hygromycin. Transformant N. benthamiana with good rooting condition was transferred to soil.

Molecular screening of transformant N. benthamiana

Genomic DNA of transformant N. benthamiana was isolated using modified CTAB method (Doyle & Doyle 1987). Isolated genomic DNA was screened by PCR amplification using Go Taq® polymerase (Promega, USA) for detection of OPKN1 gene (OP1F2: 5’ AGATATCCATTCCGGAATCTGC 3’, OP1R2: 5’ CTTCTGTCGACCCTGTCATC 3’), GUS gene (GUSF: 5’ CGCCGATGCAGATATTCGTA 3’, GUSR: 5’ ATTAAGCCTGCTGTCGAC 3’) and HPT gene (HPTF: 5’ ACAGCGTCTCCGCCGATGCA 3’, HPTR: 5’ AGTCAATGACCCTGTCGTGC 3’) together with amplification of endogenous NAD3 gene (NAD3F: 5’ TACACGGAACCTGACACT 3’, NAD3R: 5’ ATACGGAACCTGACACT 3’) as an internal control.

Gene expression profiling of OPKN1 gene in transformant N. benthamiana

Total RNA from transformant N. benthamiana was isolated using RNAzol® RT reagent (Molecular Research Center, Inc., USA). RNA with good purity and quality was converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany). The cDNA was diluted 10 times and was used as a template for analysis of OPKN1 gene expression using qPCR comparative ΔΔCt Method with normalization of geometric averaging of multiple internal control genes (Vandesompele et al., 2002). The qPCR analysis reaction was performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA) and the reaction using SensiFAST SYBR Hi-ROX Kit (Bioline, UK) based on manufacture recommendation. The expression of OPKN1 genes (KN2F: 5’ ATTCCTACTGCGGGGTCTG 3’, KN2R: 5’ GAGGTTGGTTCGTCCCTTG 3’) in transformant lines were compared to the wild type N. benthamiana and two housekeeping genes which are EF1A (EF1AF: 5’ GATTTGGGTATTGGAAAGCTC 3’, EF1AR: 5’ AGTCCATGGTGCTCCT 3’) and ACT (ACTF: 5’ TTGAAGCCTTTGCTGATGAGAGAC 3’, ACTR: 5’ GUSF: 5’ CGCCGATGCAGATATTCGTA 3’, GUSR: 5’ ATTAAGCCTGCTGTCGAC 3’, HPTF: 5’ ACAGCGTCTCCGCCGATGCA 3’, HPTR: 5’ AGTCAATGACCCTGTCGTGC 3’) together with amplification of endogenous NAD3 gene (NAD3F: 5’ TACACGGAACCTGACACT 3’, NAD3R: 5’ ATACGGAACCTGACACT 3’) as an internal control.
Phenotypic analysis of transformant *N. benthamiana*

Phenotypic comparison between *N. benthamiana* transformant and wild type was observed between 7 to 75 days after transferred into potted soil. The characterization of transformant plant was identified and measured for each line with wild type *N. benthamiana* as a comparative subject for evaluation of phenotypic characteristics. Differentiation of phenotypic characteristics between *N. benthamiana* transformant and wild type were further analysed using Microsoft Excel in order to determine the differences between transformant and wild type.

RESULTS & DISCUSSION

The *OPKN1* expression vector was successfully constructed by incorporating CaMV 35S promoter and NOS terminator flanking in between *OPKN1* gene sequence. The *OPKN1* expression cassette was then ligated into pCAMBIA 1305.2 to yield approximately 14.4 kb *OPKN1* transformation vector (pExOPKN1) (Fig. 1). pExOPKN1 transformation vector which carrying *OPKN1* gene, GUSA reporter gene and HPT selection gene was successfully transformed into *A. tumefaciens* LBA 4404.

Transformation of *OPKN1* gene via *A. tumefaciens* was conducted on *N. benthamiana*’s leaf disk and selection of transforman lines was successfully carried out with 4 lines of transformant *N. benthamiana* were regenerated (Fig. 2). The transformant plantlets of *N. benthamiana* were induced for rooting process and the plantlet successfully transferred to potted soil. In order to determine the successful *OPKN1* gene introduced into transformant *N. benthamiana*, molecular screening by PCR amplification was performed on all transformant lines. The transgenes introduced by *A. tumefaciens* infection consisting of *OPKN1*, GUSA

![Fig. 1. pExOPKN1 plasmid with OPKN1 gene expression cassette driven by CaMV 35S promoter with pCAMBIA 1305.2 as a plasmid backbone.]

![Fig. 2. In vitro selection and regeneration of transformant N. benthamiana. A) Leaf disks after infection with A. tumefaciens: pExOPKN1. B) Transformed leaf disks after 1 month in hygromycin selection media. C: 2 months of transformed leaf disks in hygromycin selection media. D) Untransformed (wild type) N. benthamiana plantlet in selection regeneration media. E) Transformed N. benthamiana plantlet in in selection regeneration media. F) Transformant N. benthamiana in potted soil.](image-url)
and HPT genes were successfully detected in all transformant lines (Fig. 3). 801 bp of OPKN1 PCR product was detected in genomic DNA sample of N. benthamiana transformant lines, T1, T2, T3 and T4. Together with PCR amplification of OPKN1 gene, PCR amplification of GUSA and HPT genes with PCR product 750 bp and 594 bp respectively in T1, T2, T3 and T4 transformant lines were also positively detected. However, no amplification for OPKN1, GUSA and HPT genes was detected in wild type N. benthamiana which act as a negative control plant.

Gene expression analysis of OPKN1 gene using quantitative Real-Time PCR (qPCR) in all transformant lines show that OPKN1 gene was overexpressed in all lines. The expression of OPKN1 gene in transformant N. benthamiana was 202.91, 94.27, 143.35 and 98.72 fold overexpressed for lines T1, T2, T3 and T4 respectively when compared to wild type plants (Fig. 4). This result indicated that the presence of OPKN1 transcript in transformant plant suggesting that the introduced OPKN1 gene cassette is transcribed to mRNA. Nevertheless, no similarity of the OPKN1 expression level between transformant lines and this difference suggesting the transgene may have different copy number and the integration site of OPKN1 gene for each line may be different in each N. benthamiana lines.

Four line of transformant N. benthamiana gave similar characteristic of flower morphology which the size of flower in transformant plant is smaller when compared to the wild type plant (Fig. 5 & Table 1). The average length of transformant N. benthamiana flower is $3.4 \pm 0.2$ cm, $3.2 \pm 0.2$ cm,
OPKN1 GENE IN N. benthamiana ALTER FLOWER SIZE AND DELAY FLOWERING TIME

Table 1. Phenotypic characterization of transformant and control N. benthamiana based on flower size and flowering time

<table>
<thead>
<tr>
<th>Lines</th>
<th>Length of flower (cm)</th>
<th>Flowering time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformant T1</td>
<td>3.4 ± 0.2</td>
<td>67</td>
</tr>
<tr>
<td>Transformant T2</td>
<td>3.2 ± 0.2</td>
<td>65</td>
</tr>
<tr>
<td>Transformant T3</td>
<td>3.3 ± 0.1</td>
<td>73</td>
</tr>
<tr>
<td>Transformant T4</td>
<td>3.2 ± 0.2</td>
<td>71</td>
</tr>
<tr>
<td>Wild type (control)</td>
<td>4.2 ± 0.2</td>
<td>59 ± 3</td>
</tr>
</tbody>
</table>

Fig. 5. Morphology comparison of transformant and wild type N. benthamiana flower. C: Flowers from wild type N. benthamiana. T: Flowers from transformant N. benthamiana transformed with OPKN1 gene.

3.3 ± 0.1 cm and 3.2 ± 0.2 cm for lines T1, T2, T3 and T4 respectively when compared to the wild type N. benthamiana with average flower length is 4.2 ± 0.2 cm. Furthermore, the transformant lines were also detected to delays flowering when compared to the wild type plants. The average period for wild type N. benthamiana to produce flower is 59 ± 3.0 days but for OPKN1 transformant lines, the duration to produce flower are 67, 65, 73 and 71 days for lines T1, T2, T3 and T4 respectively.

Productions of smaller sized flower in transformant N. benthamiana are most likely caused by the over-expression of OPKN1 gene which was transformed into N. benthamiana. The previous study reported on lettuce (Lactuca sativa) over-expressed with Arabidopsis kn1 (KNAT1) gene has produced dwarf flower with normal morphology but flowers was produced in lesser amount in transgenic lettuce (Fruis et al., 2001). Ectopic expression of rice kn1 (OSH1) gene in Arabidopsis was also produced dwarf flower in Arabidopsis but the morphology of flowers was abnormal when compared to wild type plant (Matsuoka et al., 1993).

A delay flowering in transformant N. benthamiana suggesting that the OPKN1 gene delays the plant to produce flower and this is in agreement with result obtained from over-expressing maize kn1 which caused delayed flowering in transformed maize (Bolduc and Hake, 2009).

It is suggested that high cytokinin content caused reduction of flower size and delayed flowering time in transformant N. benthamiana which over-expressing OPKN1 gene. Generally, cytokinin involved in plant cell division and high content of cytokinin was reported in transgenic plant that over-expressed kn1 gene (Ori et al., 1999). Ori et al. (1999) also reported that the characteristics of tobacco over-expressed kn1 gene were similar to the transgenic tobacco over-expressed the gene for the cytokinin production, isopentenyl transferase gene (IPT). The evidence of kn1 gene induced cytokinin content was also revealed by Sakamoto et al. (2006) on the research of kn1 protein causing the activation of isopentenyl transferase (IPT7) transcription. Instead of that, addition of high dosage of cytokinin caused reduction in bulb’s flower size, pedicel length, number of flower and also delaying the flowering time (Pobudkiewicz, 2008). Research conducted on Arabidopsis thaliana also has revealed that the accumulation of high cytokinin content through activation of genes related to cytokinin caused delayed flowering time (Igari et al., 2008).

High level of exo-cytokinin blocked the increase in length and cell number of the meristematic cells and also elongation of meristematic zone of Arabidopsis suggesting that the balancing of auxin-cytokinlin hormones may control the size of the meristem (Beemster and Baskin, 2000). Therefore, the size and the number of floral meristem are proportional to the size of
flower being produced. Based on reduction of flower size and delayed flowering time in transformant *N. benthamiana*, it is suggested that the overexpression of *OPKN1* gene increase the production of cytokinin in transformant *N. benthamiana*. However, further analysis need to be carried out in order to determine the effect of *OPKN1* overexpression on level of cytokinin content and also the possibility to study the expression profile of genes related to cytokinin production in transformant *N. benthamiana*.

**ACKNOWLEDGEMENTS**

The authors would like to thanks Malaysian Agriculture, Research & Development Institute (MARDI) and National University of Malaysia (UKM) for providing research materials and facilities.

**REFERENCES**


