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Research article

Cloning and characterization of boron transporter gene of oil palm (*Elaeis guineensis* Jacq.)

Chanakan Laksana[a], Toru Fujiwara[b,c], Sontichai Chanprame[d,e,*]

[a] *Faculty of Agricultural Technology, Burapha University Sakaeo Campus, Sakaeo 27160, Thailand*

[b] *Graduate School of Agricultural and Life Science, University of Tokyo, Tokyo, Japan*

[c] *Biotechnology Research Center, University of Tokyo, Tokyo, Japan*

[d] *Center for Agricultural Biotechnology, Kasetsart University and The Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO)/ CHE, Thailand*

[e] *Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand*

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Abstract

Boron is involved in many activities in both the vegetative and reproductive growth of oil palm. Inverse polymerase chain reaction (IPCR) based on the nested PCR method was used to clone the boron deficiency responsive gene from the genomic DNA of oil palm. The partial *EgBOR1* gene, approximately 2,000 bp, was obtained using conventional PCR and IPCR with nested PCR was used to clone the rest of the gene. The full length *EgBOR1* consisted of 3,920 bp containing 10 exons and 9 introns. The gene produced cDNA of 2,160 bp and encoded for a protein of 720 amino acid residues. Comparison of the amino acid sequence showed high similarly to the *BOR1* genes of grape, rice, maize and castor bean. The transgenic mutant yeast cell carrying *EgBOR1* showed the function of exporting boron out of the yeast cell. The gene function analysis in the transgenic *BOR1* mutant *Arabidopsis thaliana* (*bor1* mutant) carrying *EgBOR1* suggesting that *EgBOR1* gene was able to increase boron deficiency tolerance by improving root elongation under low boron supply. Analysis of *EgBOR1* gene expression showed that the gene was expressed in the roots of oil palm at a higher level than in the leaves. The results of boron uptake by mutant yeast cells transformed with *EgBOR1* and the function analysis of this gene in the *bor1*-3 mutant *A. thaliana* indicated that *EgBOR1* has a boron efflux function or that it is a boron transporter gene.

Introduction

Among the essential microelements, plants need boron (B) for growth and productivity. For example, Khan et al. (2006) found that the highest yield of wheat and rice can be achieved with the application of B at 2 kg/ha. The element is involved in many crucial metabolic activities in plants including RNA metabolism, cell wall synthesis, lignification, carbohydrate metabolism, respiration, phenol metabolism and sugar transport (Takano et al., 2010). Among micronutrient deficiencies, B deficiency is very common in arable land worldwide (Marschner, 1995) especially in high rainfall areas and calcareous soil (Shorrocks, 1997) and light textured soil (Niaz et al., 2002). *BOR1*, a boric acid/borate transporter, is a gene that regulates the transportation of boron to the xylem and causes an increment in

* Corresponding author.
E-mail address: agrstc@ku.ac.th (S. Chanprame)

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the boron concentration in shoot organs and prevents them from being boron deficient (Takano et al., 2002; Park et al., 2008). On the plasma membrane where the BOR1 protein is localized, the gene is induced to express under boron deficiency (Takano et al., 2005). The protein is internalized and moved to vacuoles and then is degraded there when the plants are under normal or high boron conditions (Takano et al., 2005). Takano et al. (2002) was the first group to identify the boron transporter, *AtBOR1* from *Arabidopsis*. Since then, more *BOR1* genes have been identified, such as *OsBOR1* from rice (Nakagawa et al., 2007) and *BnBOR1* from oilseed rape (Sun et al., 2012).

Oil palm (*Elaeis guineensis* Jacq.) is a global oil crop that is cultivated on tropical soils where rainfall is high, so that these soils easily lose their topsoil and soil fertility due to water runoff. This causes the soils contain low organic matter and plant nutrients including nitrogen, phosphorus, potassium, magnesium and boron (Goh et al., 2007). While oil palm is very sensitive to boron deficiency, on the other hand, it is highly responsive to boron application (Shorrocks, 1997). It has been shown that oil palm with severe boron deficiency symptoms produces significantly low yields (Ferwerda, 1954; Rajaratnam, 1973; Broschat, 2007; Goh et al., 2007). Sufficient boron supplementation is essential for normal growth and development throughout the plant life cycle (Kato et al., 2009). The application of boron fertilizer is a method for solving this problem but the decision to apply boron fertilizer is based on visual symptoms and so it is tricky because the symptoms of boron deficiency do not appear until 2–3 mth after the plants have been placed under stress from boron deficiency (Turner and Gillbanks, 1974).

The inverse polymerase chain reaction (inverse PCR) technique was first reported in Ochman et al. (1988). This technique provides rapid amplification of the DNA sequences that flank a known region. The primers used in this technique have their orientation in the reverse direction to those of conventional PCR. The DNA template for amplification is a restriction enzyme digestion fragment that has been ligated upon itself to form a circular DNA. The applications of the IPCR technique in molecular genetics include: the identification of the consensus sequence for insertion of transposable elements; the identification of sequences flanking transposable elements; chromosome walking; DNA library screening and full length gene cloning (Ochman et al., 1988). Therefore, the current study used the IPCR method to clone the full length of a putative boron transporter gene of oil palm and identified it by characterization of the boron efflux function of the gene.

Materials and Methods

Oil palm cultivation

Tenera oil palm (*Elaeis guineensis*) cultivar Thepha seedlings were planted in 10 cm diameter plastic pots containing vermiculite and grown under a controlled environment in a growth chamber, with a 12 hr:12 hr light:darkness regime at 28°Cand 65% relative humidity with 270 µmol/m^2/S of light intensity. They were placed on plastic trays and complete nutrition solution (CNS; Pinho et al., 2010) was added to each tray. New CNS was added every 3 d. After 4 wk, the CNS was removed and replaced by fresh CNS, boron-free CNS representing boron deficiency or CNS containing 10 mg/L of B_3HO_3 for boron toxicity. The five palm plants in each experiment were maintained under each stress conditions for 28 d.

DNA, RNA extraction and first strand cDNA synthesis

Genomic DNA and total RNA were extracted from 0.1 g of oil palm roots and leaves subjected to boron sufficient, boron deficient and boron toxicity conditions. The CTAB method was used for genomic DNA extraction. Total RNA was extracted using the method described by Laksana and Chanprame (2015). For cDNA synthesis, in 12.5 µL of total volume, the components of the reaction mixture were total RNA (1 µg), Oligo (dT_{22}) primer (2 µM), dNTP (0.8 mM) and RNase-free water. The first strand cDNA synthesis kit and protocol were from Fermentas (ThermoFisher Scientific; Lithuania).

Amplification of partial EgBOR1 gene of oil palm

The highly conserve amino acid sequences of BOR1 protein retrieved from the NCBI database were used for designing the degenerated primers. The partial length of the *BOR1* gene was amplified using PCR. The reaction mixture of 20 µL contained 100 ng of genomic DNA, 0.0125 U Ex *Taq* (Takara; Japan), 0.5X Ex *Taq* Buffer, 0.1 mM dNTP mixture and 0.15 µM of degenerated primers. Then, the PCR product was sequenced and again a pair of specific primers was designed base on the obtained sequence, namely partial *EgBOR1*F: 5'-GCACGGTC ATGGCGATATG-3' and partial *EgBOR1*R: 5'-TGGATGCAGCAGAGTATG-3'. This pair of specific primers was used to re-amplify the partial *EgBOR1*. The reaction mixture for amplification of the partial *EgBOR1* was as the same as for the former reaction. The conditions for the PCR were as described by Laksana and Chanprame (2017). The amplification products were examined using electrophoresis in 0.8% agarose gel.

Amplification of EgBOR1 gene using inverse polymerase chain reaction with nested polymerase chain reaction method

Genomic DNA (2 µg) was digested with 5U/µL of *Eco*RI (Wako; Japan) for amplification of the downstream region and *Sac*I (Takara; Japan) for amplification of the upstream region. The digestion products were incubated with 1U of T_4 DNA ligase (Takara; Japan) in a total volume of 40 µL, at 16ºC overnight to promote circularization at low concentration. The ligated DNA was purified by elution through a column (Fast Gen; Japan). The ligation products were diluted 10-fold with ddH_2O and used as the template for amplification. The sample of 20 µL of amplification mixture consisted of 0.2 mM dNTPs, 0.025 U of ExTaq DNA polymerase, 1× Ex Taq Buffer,

0.3 µM IPCR-F primer (5'-CTTGATGGGTTGTAAGCGGT-3') and IPCR-R primer (5'-CGCACCAAGCAGAAGATACA-3'). The conditions for IPCR were: preliminary denaturation at 94ºC for 1 min, then 35 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 45 s and extension at 72ºC for 1 min. The amplification products were diluted 10-fold with ddH_2O and then 1 µL was used in the second round PCR, the nested PCR with the nested-F primer (5'-TGCTTAATGACTGTCCAG-3') and the nested-R primer (5'- AGAGTGCTTGAAG AGTAC-3'). The conditions for the nested PCR were the same as for the IPCR. The nested PCR products were subjected to electrophoresis through 0.8% agarose gel. The products of IPCR and nested PCR were sequenced. The inclusion of the start and stop codons in the obtained sequence indicated that the full length of the gene had been obtained. Based on the sequences obtained, a pair of specific primers for amplification of the full length *EgBOR1* was designed as: EgFulllength F: 5'-ATGGAGGAAACATTTGTG-3' and EgFulllength R: 5'- TTACTTTGAAGTGGAGCC-3'.

Sequencing and phylogenetic analysis

The PCR products were sequenced by the Fasmac Company (Japan). The nucleotide sequences were blasted to the GenBank database (www.ncbi.nlm.nih.gov/BLAST/). The ClustalW2 program (http://www.ebi.ac.uk/tools/msa/clustalw2) was used for protein alignment between the deduced amino acid sequences from the full length *EgBOR1* and BOR1 protein of certain plant species. The MEGA-X program with 1,000 bootstraps was used for phylogenetic tree construction based on the BOR family members of *Arabidopsis thaliana*, castor bean, citrus, grape, rice, soybean and wheat.

Expression of EgBOR1 in leaves and roots of oil palm

The *EgBOR1* expression levels were investigated using real-time PCR. Samples of cDNA of oil palm after being subjected to 28 d of boron deficiency and toxicity were used for the analysis. The specific primers for the real-time analysis were *EgBOR1*RT-F: 5'-GCAGT CTGATCGAGGATTGA-3', *EgBOR1*RT-R: 5'-TGAAGCAGG TTGCTTAGTCG-3' for the *EgBOR1* gene and *Actin*RT-F: 5'-TGGTG CAGAGAGATTCAGG-3' and *Actin*RT-R: 5'-AGGGAACATGGTT GATCCTC-3' for the *EgActin* gene. The real-time PCR was performed with three biological and three technical replications. The *EgActin* gene was used for normalization.

Expression of EgBOR1 in yeast

The *Saccharomyces cerevisiae* stain Y01169 (*bor1*Δ) (*MATa his*3 *leu*2 *met*15 *ura*3 *YNL275W*::*KANmx*4) was used as this mutant yeast stain accumulates a higher protoplasmic boron concentration than the wild type stain under a normal concentration of boron (0.5 mM H_3BO_3) (Takano et al., 2002). The plasmid pYES2, pYES2 containing *AtBOR1* (the *Arabidopsis* boron transporter) gene and pYES2 containing the *EgBOR1* gene were transformed to *S. cerevisiae* and then incubated in synthetic minimal glucose medium (Sherman, 2002) containing 2% galactose and 0.5 mM H_3BO_3 at 30°C for 1 hr. Measurement of boron uptake was performed using the method described by Nozawa et al. (2006). The means of boron concentration were separated using least significant differences at $p < 0.05$.

Characterization of EgBOR1 in bor1 mutant Arabidopsis thaliana

EgBOR1 was inserted into plasmid pMDC 32 which contained 2x35S promoter for driving the expression of *EgBOR1* and the hygromycin-resistant gene, a selectable marker gene, to get the recombinant plasmid pMDC32-*EgBOR1*. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90). Then, the *bor1* mutant *A. thaliana* (requiring a high level of boron) was infected with this *Agrobacterium* using the floral dip method (Zhang et al., 2006). The seeds of the infected *bor1* mutant plant were germinated on Murashige and Skoog solid medium supplemented with 20 µg/mL hygromycin (Merk; China) plus 250 µg/mL cefotaxime (Claforan; Sanofi Aventis; Tokyo, Japan). After 7–10 d on selective medium, the seedlings with healthy green cotyledons and true leaves were identified as the transformants. Then, the transformed seedlings were transferred to MGRL medium (Fujiwara et al., 1992) containing 0.03 µM B_3HO_3 (boron deficient) for determining the root morphology and confirming the integration of the transgene.

Results

Amplification of partial and full length of EgBOR1 gene of oil palm

Amplification of the partial *EgBOR1* gene was carried out using the degenerated primers. A DNA fragment size of approximately 2,000 bp was obtained from conventional PCR. The obtained nucleotide sequence showed high similarity to the boron transporter gene of many plant species. Thus, this DNA fragment was appropriate for designing the primers for IPCR with nested PCR to obtain the full length of the oil palm *EgBOR1* gene.

For the IPCR with nested PCR, the genomic DNA extracted from the root of a boron-deficient oil palm plant was separately digested with two different restriction enzymes. The self-ligation of *Eco*RI digested fragments was used for downstream region amplification, with the reaction yielding a DNA fragment of approximately 1,700 bp. This fragment contained about 400 bp of the *EgBOR1* gene including the stop codon. The self-ligation of *Sac*I digested fragments was used for upstream region amplification. The PCR product obtained was approximately 2,500 bp. This fragment contained 1,500 bp of the *EgBOR*1 gene including the start codon. In total, the full length of the *EgBOR1* gene derived from the genomic DNA of oil palm using the IPCR technique was approximately 3,920 bp, which contained 10 exons and 9 introns. The full length cDNA of *EgBOR1* gene was

sequenced and revealed that the ORF of this gene was 2,160 bp (Accession: KP231205.1) and encoded 720 amino acid residues. Alignment of the deduced amino acid of this protein showed high similarity to the BOR1 protein of many plants species such as VvBOR2 of grape (XP_002272575.1, 79%), CmBOR1 of citrus (ABQ52428.1, 82%), OsBOR1 of rice (AAQ02664.1, 85%) and AtBOR1 of *Arabidopsis thaliana* NP_850469.1, 81%), as shown in Fig. 1 and Fig. 2.

Transcription levels of EgBOR1 in leaves and root of oil palm

The relative expression levels of the *EgBOR1* gene in the roots and leaves of oil palm were investigated using real-time PCR after being subjected to 28 d of boron deficiency and boron toxicity. The reference gene was the *Actin* gene. The relative expression of the gene was compared with the control condition (0 d). The results showed that the *EgBOR1* gene expression in boron deficiency stress was higher than that of the boron sufficient and toxicity conditions. The expression of the gene in roots subjected to boron deficiency was about three times higher than in the leaves. Furthermore, under the boron deficiency condition, the expression levels of the gene in the leaves and roots were higher than and significantly different compared especially to the boron toxicity condition (Fig. 3).

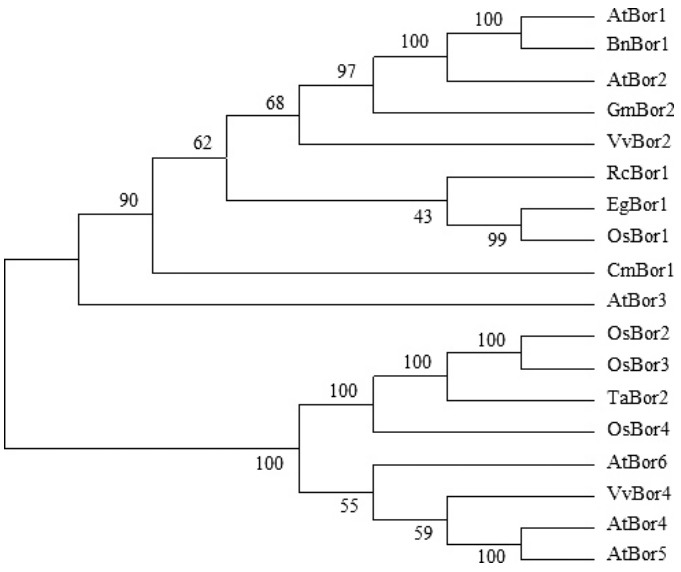

Fig. **1** Phylogenetic tree construction of deduced amino acid sequences of oil palm (EgBor1), *Arabidopsis thaliana (*AtBor1– 6*)*, castor bean (RcBor1), citrus (CmBor1), grape (VvBor2,4), rapeseed (BnBor1), rice (Os1– 4), soybean (GmBor2) and wheat (TaBor2) based on neighbour-joining using MEGA-X program**.** The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

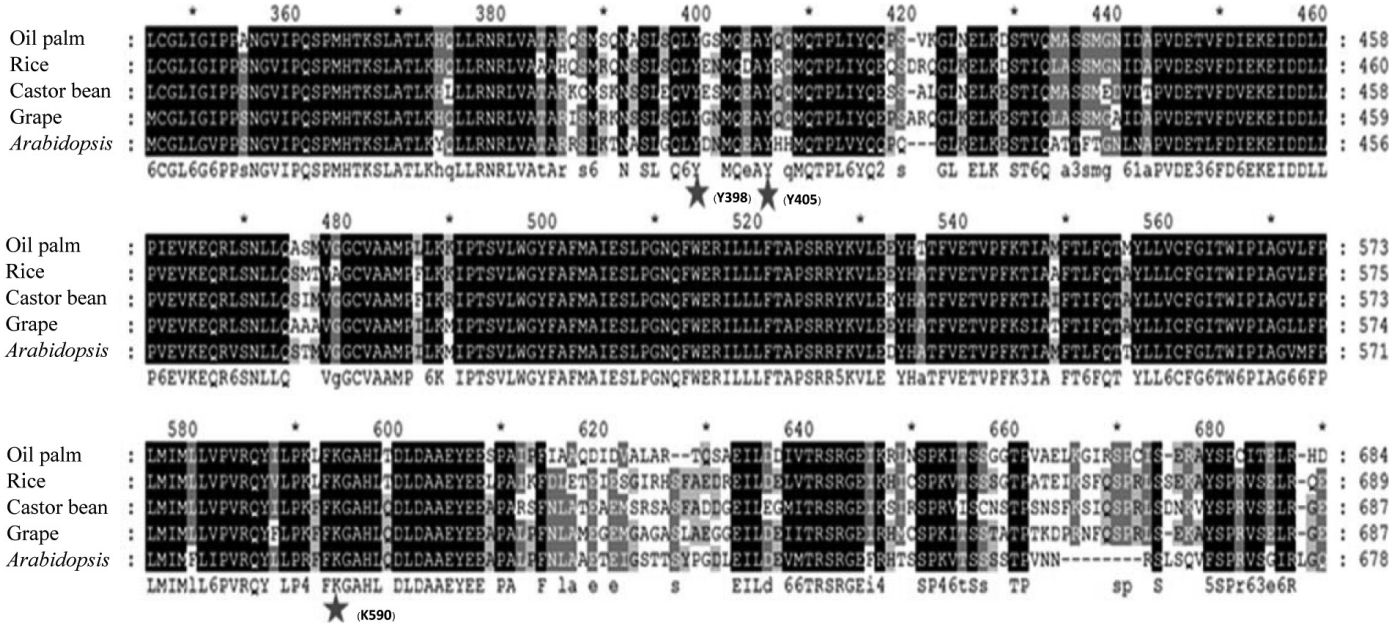

Fig. **2** Comparison of amino acid sequence of EgBOR1 with rice; OsBOR1 (AAQ02664.1), castor bean; RcBOR1 (XP_002519293.1), grape; VvBOR2 (XP_002272575.1) and *Arabidopsis*; AtBOR1 (NP_850469.1) for identifying key residues involved in polar localization (★ = key residue)

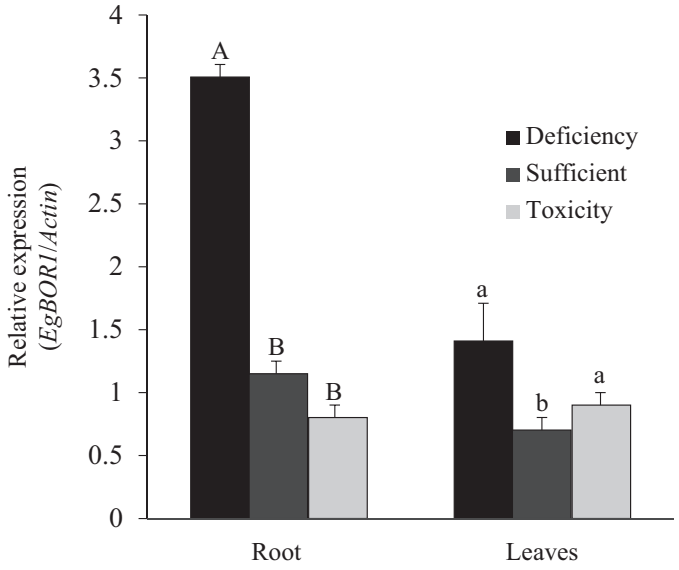

Fig. **3** Relative expression level of *EgBOR1* in roots and leaves of oil palm subjected to 28 d of boron deficiency, sufficiency and toxicity conditions, where same letter above columns denotes non-significant ($p < 0.05$) difference.

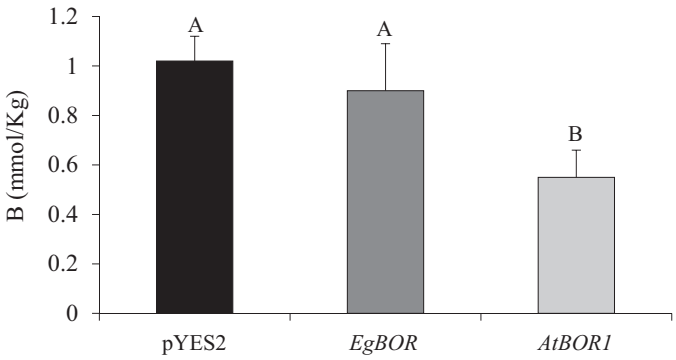

Fig. **4** Boron concentrations in three mutant Y01169 (*bor1*Δ) yeast cells transformed with pYES2 (pYES2 backbone), *EgBor1* (pYES2 carrying *EgBor1*) and *AtBor1* (pYES2 carrying *AtBor1*), where same letter above columns denotes non-significant ($p < 0.05$) difference and error bars = + SD

Expression of EgBOR1 in yeast

The activity of *EgBOR1* was determined in the mutant Y01169 (*bor1*Δ) yeast strain to ensure that *EgBOR1* is an efflux boron transporter. The result indicated that the concentration of boron in the yeast cell carrying *EgBOR1* was 12% lower than for the mutant cell carrying a control vector (the pYES2 vector without *EgBOR1*), while the yeast cell containing *AtBOR1* could export boron out of the cell based on the result of the 50% lower boron concentration compared to the mutant yeast strain (Fig. 4).

Characterization of EgBOR1 in bor1 mutant Arabidopsis thaliana

The gene was transformed into the *bor1* mutant *A. thaliana* to determine the function of the *EgBOR1* gene in the plant system. The transgenic *A. thaliana* seedlings were cultured in MGRL medium containing 0.03 µM H_3BO_3 for 5 d. This boron concentration is considered to produce boron deficiency in the *bor1* mutant *A. thaliana*. The results showed that the roots of the transgenic seedlings were healthy and had better growth compared to both the wild type and the original mutant seedlings. The transformed seedlings had more lateral roots that were longer than for the non-transformed seedlings. However, the wild type seedlings had better growth of the aerial part than the transgenic seedlings, with the latter being short with leaves that had not expanded very well (Fig. 5).

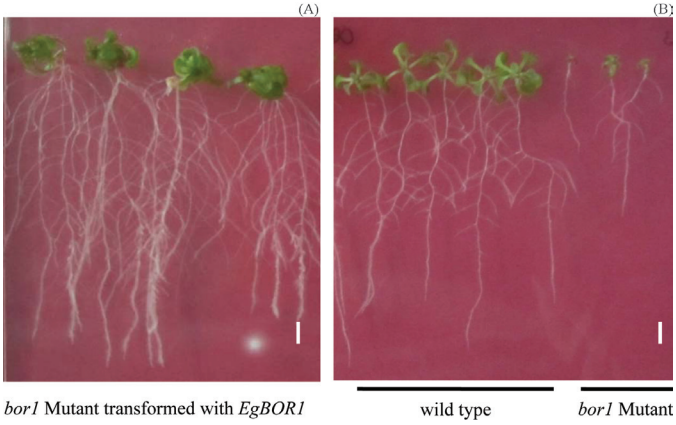

Fig. **5** Effect of *EgBOR1* in *bor1* mutant *A. thaliana, where EgBOR1* restores phenotype of *bor1* mutant (A) under boron deficiency condition compared to wild type and *bor1* mutant *A. thaliana* (B) and white scale bar = 1 cm

Discussion

The current study involved using IPCR with nested PCR to clone the *EgBOR1* gene from oil palm root subjected to boron deficiency conditions. Boron contributes to cell wall synthesis through cross-linking the pectin polysaccharide rhamnogalacturonan II in the primary cell wall (Miwa and Fujiwara, 2010). The *BOR1* gene has been successfully isolated from many plants such as *Arabidopsis thaliana* (Takano et al., 2002), *Oryza sativa* (Nakagawa et al., 2007), oilseed rape (Sun et al., 2012), grape (Pérez-Castro et al., 2012)

and *Brassica napus* (Chen et al., 2018). The current study reports on using *EgBOR1* as the first boron transporter in oil palm, using the IPCR method for the rapid amplification to obtain the flanking regions of the known sequence (Ochman et al., 1988). Although the first round of upstream region amplification was successful, the IPCR product was not unique (data not shown)as it was composed of many DNA fragments because of the complexity of the genomic DNA or the self-ligation random events. Then nested PCR was performed and obtained a single band of DNA. The analysis of the phylogenicity of the amino acid sequence deduced from the cDNA of *EgBOR1* and the amino acid sequences of many plant species revealed two groups of gene. *EgBOR1* was in the group of boric acid/borate exporters that regulate efficient boron transportation to the xylem (Takano et al., 2002). This group contains *OsBOR1*, *VvBOR1*, *RcBOR1*, *AtBOR1*, *AtBOR2*, *AtBOR3*, *BnBOR1*, *GmBOR2* and *CmBOR1*. The other group contains *TaBOR2* (Reid, 2007), *OsBOR2*, *OsBOR3*, *OsBOR4*, *VvBOR4*, *AtBOR 4* (Miwa et al., 2007), *AtBOR5* and *AtBOR6*.

In addition, EgBOR1 had tyrosine signals (Y398 and Y405) compared with AtBOR1 (Fig. 2). Both positions are tyrosine-based signals in endocytic degradation and have polar localization of BOR1 (Takano et al., 2010; Yoshinari et al., 2012). Furthermore, the lysine position is very important in BOR1, as the Lys-590 position of AtBOR1 is required for boron-induced ubiquitination and boron-induced endocytic degradation (Kasai et al., 2011). EgBOR1 is conserved at the same position as AtBOR1 (K590). The endocytic pathways regulating BOR1 include the localization of the protein in the plasma membrane and degradation in the vacuole (Yoshinari et al., 2019).

BOR1 also acts as a boron efflux transporter and is involved in boron export (Nozawa et al., 2006). In the *Arabidopsis*, BOR1 is a borate exporter that facilitates xylem loading of boron under boron deficiency conditions (Aibara et al., 2018). When the *AtBOR1* was expressed in yeast, it decreased boron concentrations in the yeast cells (Takano et al., 2002). In the current study, when the yeast cell was transformed with *EgBOR1*, it performed a similar function of exporting boron out of the yeast cell, as *AtBOR1* did. However, the boron transport efficiency of *EgBOR1* was about 12% lower and it was significantly different when compared with *AtBOR1*. This result suggested that *EgBOR1* acts as a boron exporter but with a lower efficiency than *AtBOR1*. This result was as the same reported for *VvBOR1* of grape, which was reported to export boron at a lower efficiency than *AtBOR1* (Pérez-Castro et al., 2012). The current study confirmed the expression of the *EgBOR1* gene using real-time PCR. Under the boron deficiency condition, the relative expression level of *EgBOR1* was higher than that for boron toxicity. This result also corresponded with a study in *Arabidopsis* where *AtBOR1* was up-regulated under the limitation of boron supply and when the boron was resupplied, BOR1 was degraded (Takano et al., 2005). Chen et al. (2018) also found that the expression of *BnBOR1;1c* from *Brassica napus* was up-regulated by boron deficiency. Aibara et al. (2018) reported that boron toxicity was avoided by the regulation of *AtBOR1* which depended on the concentrations of boron. With a boron deficiency, the BOR1 protein was accumulated and the protein was degraded in sufficient boron conditions. Under a toxic boron condition, the synthesis of BOR1 protein was stopped via translation suppression.

pMDC32, containing *EgBOR1* driven by the 2×35S promoter, was introduced into the *Agrobacterium tumefaciens* stain GV3101 and then transformed to the *bor1* mutant *A. thaliana*. On selective medium containing 20 µg/mL hygromycin, the transformed plants were easily distinguishable as healthy seedlings with normal roots, cotyledons and true leaf development, while non-transformed seedlings had smaller cotyledons than the true transformants. The root development was normal only in true transformants. The true transformed *A. thaliana* seedlings were transferred to MGRL medium supplement 0.03 µM H_3BO_3. The results showed that they were healthy and had better growth compared to both the wild type and the mutant plants. Roots of the transformed seedlings showed more lateral rooting and were longer than the wild and mutant types. However, in the current study, the growth of the aerial part of transgenic seedlings was less than in the wild type. They were short and the leaves were not very well expanded. Nakagawa et al. (2007) reported that the absorption of boron was slightly different in the transport mechanism of boron between monocotyledonous and dicotyledonous plants. *A. thaliana* is a dicotyledonous plant while oil palm is monocotyledonous plant and both nucleotide and amino acid sequences of *EgBOR1* and *AtBOR1* are different. These differences may cause an improper expression of *EgBOR*1 in the transformed *Arabidopsis*.

IPCR with nested PCR was highly efficient for the rapid and cost effective cloning of the full-length cDNA of a gene. This method was used for cloning the full length *EgBOR1* cDNA from oil palm (*Elaeis guineensis* Jacq.). Although the subcellular localization of the gene was not available, the function of the gene as a boron transporter gene was implied by the results of boron efflux in transgenic yeast and root growth restoration of transgenic *Arabidopsis* in a boron deficiency condition including conservation of tyrosine-based signals in endocytic degradation and polar localization. The identification of this gene will improve understanding of the conditions concerning boron metabolism and boron metabolism as they relate to gene expression. The relationship between the expression patterns of the gene under different boron conditions and the accumulation of boron in the shoots of different oil palm cultivars will be the next step to study. If there is a significant difference in the expression levels of the gene between B-efficient and B-inefficient oil palm cultivars, the gene expression levels can be used as a simple indicator for the selection of boron deficiency tolerance in oil palm cultivars.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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