CLONING OF *BOR1* (BORON TRANSPORTER) PARTIAL LENGTH CDNA FROM OIL PALM, SUGARCANE AND PHYSIC NUT

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(Received: March 28, 2014; Accepted: September 29, 2014)

ABSTRACT

Boron is a micronutrient that plants need in small amounts but very vital for plant development. Boron deficiency in plant is a commonly found in Thailand. If the genes that regulate the boron transport in plant are known, this will lead to a better understanding of boron metabolism and boron supplementation can be managed effectively. This study was aimed to obtain the fragment of the *BOR1* cDNA using RT-PCR. Total RNA from young leaves of oil palm, physic nut and sugarcane were extracted, respectively, and used as template for cDNA synthesis by RT-PCR. The PCR fragments about 300 bp of cDNA derived from primer specific to *BOR1* cDNA in the studied plant species were amplified and sequenced. The cDNA sequences shared a similar homology to *BOR1* of many plants species such as castor bean, *Fragaria vesca*, soybean, grape, *Brassica napus* and *Arabidopsis*. It can be concluded that the synthesized cDNA is a part of *BOR1* cDNA of the corresponding plant species. Analysis of *BOR1* cDNA expression in leaves and roots of oil palm, sugarcane and physic nut by real-time PCR showed that the *BOR1* cDNA expression in roots was higher level than that in leaves of those plant species.

Key words: RNA extraction, RT-PCR, real-time PCR, gene expression, boron deficiency

INTRODUCTION

Boron is essential for plant growth (Takano *et al*., 2010). Boron involves in many activities in plant such as sugar transport, RNA metabolism, cell wall synthesis, lignifications and carbohydrate metabolism (Franco *et al*., 2011). Most of boron is localized in cell wall of plant. Boron deficiency is one of the most common and widespread of all nutrient deficiencies (Marschner, 1995; Takano et al. 2005). It occurs in high rainfall areas, calcareous soil, soils with high clay content and low fertility soils (Shorrocks, 1997). The boron requirement of plant is low but significantly different among

various species (Sun et al. 2012). Under boron limitation, *BOR1* expression is induced and localized to the plasma membrane (Takano et al. 2005). *BOR1* is a boric acid or borate transporter for xylem loading and increases the concentrations of boron in xylem sap and shoot organs, thereby acting to protect the shoot from boron deficiency (Takano et al. 2002). *BOR1* is a transporter gene whose transcript is accumulated and required for efficient boron translocation under boron limited conditions (Takano et al. 2002; Takano et al. 2005). *AtBOR1* is the first boron transporter gene had been identified from *Arabidopsis*. *AtBOR1* is a boron transporter mediating boron exports from pericycle cells into root stellar apoplast against a concentration gradient (Takano *et al*., 2002). Moreover, *BOR 1* of many plant species have been identified so far such as rice (*OsBOR1*) (Nakagawa et al. 2007) grape (*VvBor1*) (Pérez-Castro et al. 2012) and citrus (Canon et al. 2013).

Oil palm, sugarcane and physic nut are important economic and biofuel crop. Oil palm is one of 16 plant species most sensitive to boron deficiency and highly responsive to boron application (Shorrocks, 1997). The symptoms of boron deficiency in oil palm are crinkle leaf, hook leaf, small crumpled new leaves, blind leaf, rounded frond tip, white stripe and leaflet shatter (Turner and Gillbacks, 1974). Julrasak (2006) collected 860 samples of oil palm plants from the field in south of Thailand and determined the boron concentration in the leaves. The results showed that 233 samples were severe boron deficiency. Under severe symptom, it causes yield decrease and low percentage of palm oil. Boron deficiency reduces not only yield but also the quality of palm oil (Tanaka and Fujiwara, 2008). In sugarcane, the primary role of boron is a key player in metabolism of carbohydrates and transportation of sugars through membranes which is highly important for sugarcane production (Huang *et al*., 2007). When sugarcane grows under low boron, it shows malformed phenomenon such as chlorosis of immature leaves, distorted leaves particularly along the leaf margins of immature leaves. The apical meristem may die, young sugarcane plants tend to be brittle and bunched with many tillers (Mabry et al. 2013). Physic nut is one of the most promising oil crop with high quality of oil for biodiesel but very few investigation in boron. Santos et al. (2013) reported that under low boron deficiency in physic nut, dry matter production was reduced. Normally, oil palm is grown in high rain fall area and low soil boron contents (Shorrocks, 1997). Sugarcane and physic nut are grown in unsuitable areas and low soil fertility such as light texture soils, calcareous soils and intensively cultivated soils (Madhuri et al. 2013). All of these soils are limited in micronutrients content especially boron which is required for sugar transport, RNA metabolism, cell wall synthesis, lignifications, cabohydrate metabolism (Marchner, 1995).

Treatment method for boron deficiency is the application of fertilizer with boron or application of boron directly to the plants. The requirement of this element in plants is in a narrow range between boron deficiency and toxicity (Chapman et al. 1997). This makes it quite difficult to manage. However, if the expression of genes related to boron metabolism is well understood, it will facilitate boron management more efficiently in the plants. Thus, this research was aimed to clone partial length of *BOR1 cDNA* from oil palm, sugarcane and physic nut and study the expression of the gene under boron deficiency. This will be the starting point for understanding boron metabolism related gene which will eventually lead to better boron management and/or plant breeding program for boron deficiency tolerant cultivars.

MATERIALS AND METHODS

Plant samples and RNA extraction

Seedlings of oil palm, sugarcane and physic nut were grown in vermiculite. These plants were applied with MS medium under boron deficient condition (no boron supplemented). Leaves and roots were collected at 0, 7, 14 and 21 days of boron deficiency treatment for RNA extraction. Total RNA was extracted from 0.1 g of leaves and root by the method described previously by Laksana (2011).

For cDNA synthesis, the reaction mixture consisted of 1 µg of total RNA sample, 2µM Oligo (dT) primer, 0.8mM dNTP. RNase-free water was added to make up a final volume of 12.5 µl and mixed gently. The reaction was incubated at 65ºC for 5 min and then cooled at 4ºC for at least 2 min. After that, 1x reaction buffer, 0.5U RiboRock RNase inhibitor (Fermentas), 1mM dNTP and 1 µl Revert Aid M-MuLVRT (Fermentas) were added in the reaction tube, mixed gently and incubated at 42ºC for 1 hr and stopped the reaction at 70ºC for 10 min then cooled at 4ºC for at least 2 min. RNaseH (0.2 µl) was added for removing of the remained total RNA.

Amplification of partial length *BOR1* cDNA fromoil palm, sugarcane and physic nut

Primers used in this study are listed in Table1. The specific primers were designed based on the highly conserve region of amino acid sequence deduced from BOR1s sequences of NCBI database. The amplification reaction was carried out in a total volume of 20 µl, with 100 ng of cDNA, 1 µl of 1mM dNTPs, 1U of *Taq* polymerase (5u/µl) (Fermentas, Canada), 4 µl of 25 mM of $MgCl_2$, 2 µl of 10x buffer (Fermentas, Canada), and 0.5 µl of 5 µM of forward and reverse of each primer (Table 1). The amplification was performed under the following conditions: preliminary denaturation at 95 °C for 3 min; then 30 cycles of denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72°C for 5 min. The amplification product was resolved on a 0.7% (w/v) agarose gel electrophoresis at 100 V for 40 min.

Sequencing and phylogenetic analysis

The PCR products were eluted from the 0.7% (w/v) agarose gel using the PCR cleanup and gel extraction kit (NucleoSpin® Extract II) following manufacturer's protocol and were sequenced by the First Base Laboratory (Malaysia). The sequences were compared to GenBank databases (www.ncbi.nlm.nih.gov/BLAST/) and translated into amino acid sequence using Genetyx program. The derived BOR1 amino acid sequence was aligned to BOR1 amino acid sequences of many plant species by the ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2). A phylogenetic tree, based on BORs family members from *Arabidopsis*, rice, grape, soybean, wheat, citrus and caster bean were analysed using Phylogenetic program (http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId =clustalw2-I20140311-230120-0632-88959908-oy&analysis=phylotree).

Expression of *BOR1* cDNA in leaves and roots of oil palm, sugarcane and physic nut

BOR1 expression level was investigated by real-time PCR quantitative. The specific primers for *BOR1* were designed from the cloned partial length *BOR1* cDNA of the three plant species using primer3 program (http://simgene.com/Primer3). Primers used in this study are listed in Table 2. The product sizes were about 150 bp for oil palm and sugarcane and 120 bp for physic nut. The cDNA from oil palm, sugarcane and physic nut derived from boron deficiency for 0, 7, 14 and 21 days were used for analysis. The amplification reaction was carried out in a total volume of 20 µl, with 100 ng of cDNA, 10 µl 2x SensiFAST SYBR No-ROX Mix buffer (Bioline Reagent Ltd.) and 0.8 µl of 10µM of forward and reverse of each primer. The amplification was performed under the following conditions: preliminary denaturation at 95 °C for 30 s; then 45 cycles of denaturing at 94°C for 5 min, annealing at 58°C for 15 min, and extension at 72°C for 10 min. Real-time PCR was conducted using Mastercycler® ep realplex4 from Eppendorf®. The expression of these genes were compared with control condition (0 day) and the reference gene was *Actin* gene for oil palm and sugarcane and *Catalase* gene for physic nut. *Actin* is considered to be reasonable reference gene due to it constitutive expression and the most commonly used reference genes (Huggett et al. 2005). Three biological replicates of the real-time PCR analysis and qRT-PCR was carried out in three technical replicates for each sample.

RESULTS AND DISCUSSION

Cloning and molecular characterization of BOR1 partial length cDNA from oil palm, sugarcane and physic nut

Boron is one of micronutrient that essential for plant growth when boron in soil is low, the plants are subjected to boron deficiency which in turn induces the *BOR1* expression. *BOR1* is a boron transporter located in plasma membrane of root pericycle (Takano et al. 2002). In the present study, the partial of *BOR1* cDNAs of oil palm, sugarcane and physic nut were identified. Total RNA was isolated from leaves and roots and then transcribed to cDNA. The specific primers (Table1) were designed based on conserved amino acid sequences of BOR1 protein of many plants species. The PCR products size of 298, 293 and 311 bp were obtained from oil palm, sugarcane and physic nut, respectively (Fig. 1).

Table 1. Primers for amplifying of *BOR1*, *Actin* and *Catalase* cDNA.

Remarks	Primers	Sequence
For amplifying partial *Bor1* of oil palm, sugarcane and physic nut	*patialBor1*F	5'-CATACTCTGCTGCATCCA-3'
	*patialBor1*R	5' -GTCCTCTGGGGTTACTTT-3'
For amplifying *Actin* (internal control for oil palm and sugarcane)	*Actin*F	5'-CATGCCATCCTTCGATTGG-3'
	*Actin*R	5'-CACATCTGCTGGAAGGTGC-3'
For amplifying *Catalase* (internal control for physic nut)	*CAT*F	5'-CCGGTGATTGTCCCTTTCTCCAC-3'
	*CAT*R	5'-AATATTCCTGTTCACACCAACCG-3'

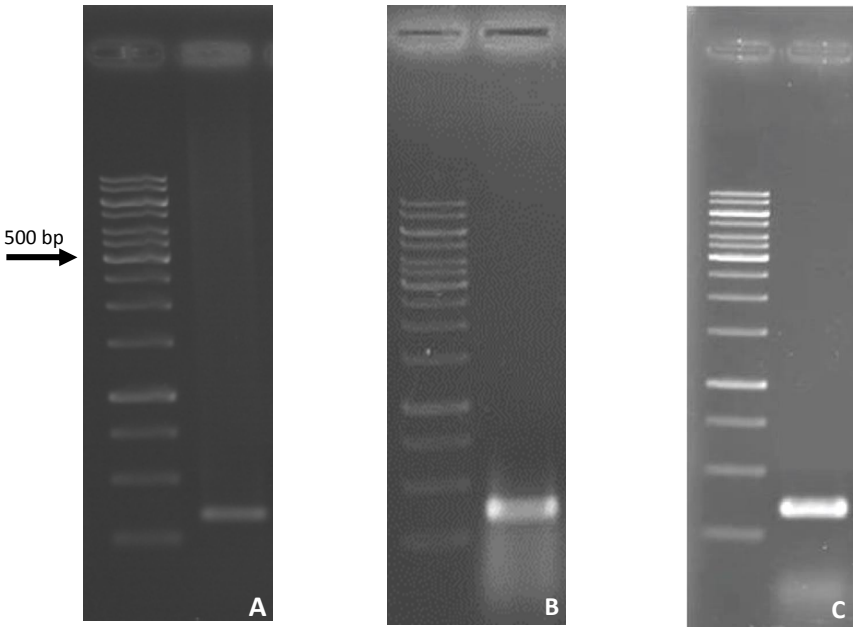

Fig. 1. The PCR product derived from PCR reaction with primers specific to *BOR1* cDNA. Partial of *BOR1* cDNA of oil palm (A), sugarcane (B) and physic nut (C)**.**

These fragments were sequenced and blasted in NCBI. The results showed that the fragments were homologous to *BOR1* cDNA of many other plant species such as castor bean (XM_002519247.1), *Fragaria vesca* (XM_004297058.1), soybean (XM_003554519.2), grape (NM_001280891.1) *Brassica napus* (GU827643.1) and *Arabidopsis* (AB073713.1). The nucleotide sequences of PCR product derived from sugarcane and physic nut showed similarity to *AtBOR1* of *Arabidopsis* (Fig. 2).

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Physic          --GTCCTCTGGGGTTACTTTGCCTTCATGGCCATAGAAAGCTTACCTGGAAACCAGTTCT 58
Oil             --GTCCTCTGGGGTTACTTTGCCTTCATGGCCATAGAAAGCTTACCTGGAAACCAGTTCT 58
sugarcane       --GTCCTCTGGGGTTACTTTGCCTTCATGGCCATAGAAAGCTTACCTGGAAACCAGTTCT 58
Grape           CAGTTCTTTGGGGCTATTTTGCTTTCATGGCGATTGAAAGCTTGCCTGGAAATCAGTTCT 69
Casterbean      CAGTTCTGTGGGGCTATTTTGCTTTCATGGCCATTGAAAGCTTACCTGGTAACCAGTTCT 443
soybean         CTGTACTTTGGGGCTATTTTGCTTTGATGGCCATTGAAAACTTACCTGGCAACCAGTTTC 443
Brassica        CTGTCCTTTGGGGTTACTTCGCCTTCATGGCTATTGAGAGCTTACCTGGAAACCAGTTCT 69
Arabidopsis     CAGTCCTTTGGGGCTATTTTGCCTTCATGGCCATCGAAAGCTTACCCGGAAACCAATTCT 69
Fragaria        CAGTCCTCTGGGGCTACTTCGCTTTCATGGCCATTGAAAGCTTACCGGGTAACCAATTTT 780
                  ** ** ***** ** ** ** ** ***** ** ** * *** ** ** ** ** **

Physic          GGGAGAGGATTCTCTTGCTCTTCACCGCACCAAGCAGAAGATACAAAGTGCTTGAAGAGT 118
Oil             GGGAGAGGATTCTCTTGCTCTTCACCGCACCAAGCAGAAGATACAAAGTGCTTGAAGAGT 118
sugarcane       GGGAGAGGATTCTCTTGCTCTTCACCGCACCAAGCAGAAGATACAAAGTGCTTGAAGAGT 118
Grape           GGGAGAGAATTCTGTTACTTTTCACTGCTCCAAGTCGAAGATACAAGGTGTTGGAGGAGT 129
Casterbean      GGGAGCGGATCTTGTTGCTTTTCACTGCTCCAAGCAGGAGATACAAAGTGCTGGAGAAAT 503
soybean         GGGAATGGATTTTATTAATTTTCATTGCTCCAAGTCGAAGATACAAAGTCTTGGAGGAGT 503
Brassica        GGGAGAGAATCTTACTTCTCTTCACCGCCCCAAGTCGCCGCTTCAAGGTTCTTGAAGATT 129
Arabidopsis     GGGAAAGAATCTTACTTCTGTTCACCGCCCCAAGTCGCCGCTTCAAGGTTCTTGAAGATT 129
Fragaria        GGGAAAGGATCTTATTGCTCTTTACTGCTCCCAGTAGAAGATACAAAGTCCTCGAGGAGT 840
                ****  * **  *  *  * ** *  ** ** **  *  * * *** **  * **  * *

Physic          ACCATGCCACCTTCGTGGAGACTGTACCTTTCAAGACAATTGCTGCTTTTACCCTTTTCC 178
Oil             ACCATGCCACCTTTGTGGAGACTGTACCTTTCAAGACAATTGCTGCTTTTACCCTTTTCC 178
sugarcane       ACCATGCCACCTTCGTGGAGACTGTACCTTTCAAGACAATTGCTGCTTTTACCCTTTTCC 178
Grape           GTCATGCAACATTTGTTGAGACTGTGCCATTCAAAGCAATTGTCACCTTCACCTTGTTCC 189
Casterbean      ACCATGCCACCTTTGTGGAAACTGTGCCTTTCAAGACGATTGCAATATTTACGATTTTTC 563
soybean         GCCATGCAACTTATGTGGAAACCGTACCATTCAAGACAATTGCAGTATTCACAGCCTTCC 563
Brassica        ACCACGCAACGTTCGTTGAGACGGTTCCATTCAAGACGATTGCGATGTTCACTATTTTCC 189
Arabidopsis     ACCACGCGACATTCGTGGAAACCGTTCCATTCAAGACGATTGCAATGTTTACTCTTTTCC 189
Fragaria        ACCATGCAACTTTTGTAGAAACTGTGCCTTTCAAGACAATTGTGATGTTTACAATTTTCC 900
                  ** ** ** *  ** ** ** ** ** *****  * ****     ** **    ** *

Physic          AGACCGCTTACTTGCTATTGTGCTTTGGAATAACATGGATTCCTATAGCCGGGGTCCTCT 238
Oil             AGACCGCTTACTTGCTATTGTGCTTTGGAATAACATGGATTCCTATAGCCGGGGTCCTCT 238
sugarcane       AGACCGCTTACTTGCTATTGTGCTTTGGAATAACATGGATTCCTATAGCCGGGGTCCTCT 238
Grape           AGACGGCCTACTTGCTTGTGTGTTTTGGTATAACATGGATCCCAATAGCTGGGGTCCTTT 249
Casterbean      AGACAGCTTACTTGCTGGTTTGTTTTGGCATTACATGGATTCCAATTGCTGGGGTTCTAT 623
soybean         AGACTGCTTACTTGCTTGTTTGTTTCGGTATCACTTGGGTGCCAATAGCTGGGGTTCTAT 623
Brassica        AAACGGTTTATCTGTTAATCTGCTTTGGCCTCACATGGATCCCAATAGCAGGAGTCATGT 249
Arabidopsis     AAACGACTTATCTCTTGATCTGCTTTGGTCTCACATGGATACCAATCGCAGGAGTCATGT 249
Fragaria        AAACTGTGTACTTGCTTATATGTTTTGGGCTTACTTGGGTTCCAATTGCCGGGGTCATGT 960
                * **    **  *  *  * ** ** **  * ** *** * ** ** ** ** **  * *

Physic          TTCCATTGATGATCATGCTTTTGGTTCCTGTGAGACAGTATGTTCTCCC--AAGCTTTCA 296
Oil             TTCCATTGATGATCATGCTTTTGGTTCCTGTGAGACAGTATGTNNTNCC--AAGCTCTAA 296
sugarcane       TTCCATTGATGATCATGCTTTTGGTTCNNGNCAGCTCGAGTATGTTNNT--NNGC----A 292
Grape           TCCCAATGATGATCATGCTTCTCGTTCCAGTACGGCAGTATTTGCTCCCCAAATTTTTCA 309
Casterbean      TCCCGTTAATGATCATGCTTTTGGTTCCTGTGAGACAATACATTTTGCCCAAGTTCTTCA 683
soybean         TCCCATTGATGATCATGCTTCTGGTTCCTGTAAGGCAATACATTCTGCCCAAGTTTTTCA 683
Brassica        TCCCTTTAATGATCATGTTCTTAGTCCCCGTAAGACAATACATCCTCCCTAGATTCTTCA 309
Arabidopsis     TCCCTTTAATGATCATGTTCTTAATCCCCGTACGACAATATCTCCTCCCTAGATTCTTCA 309
Fragaria        TTCCTTTGATGATCATGCTTTTGGTCCCTGTGAGACAATACTTTCTGCCCAAGTTTTTCA 1020
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Fig. 2. The alignment result of cDNA fragment derived from oil palm, sugarcane and physic nut to *BOR1* of other plant species using clustalW2 program.

Takano et al. (2002) reported that *AtBOR1* is an efflux-type boron transporter for xylem loading and is essential for protecting shoots from boron deficiency. This suggests that cloned cDNA fragments from the three plant species may be a boron transporter gene responsible for transportation of boron in plant. This is a first study to report on identification of boron transporter cDNA in oil palm, sugarcane and physic nut so called *EgBOR1*, *ScBOR1* and *Jc BOR1*, respectively.

Phylogenetic analysis

The nucleotides of three putative *BOR1* cDNA were translated into amino acid sequences for phylogenetic analysis with BOR1 protein of many plant species. The BOR1 proteins could be classified into 2 groups. EgBOR1 (oil palm), ScBOR1 (sugarcane) and JcBOR1 (physic nut) belong to the group of boric acid/borate exporter. This group contains AtBOR1, BnBOR1, AtBOR2, GmBOR2, VvBOR1, CmBOR1,RcBOR1 and OsBOR1 (Fig. 3).

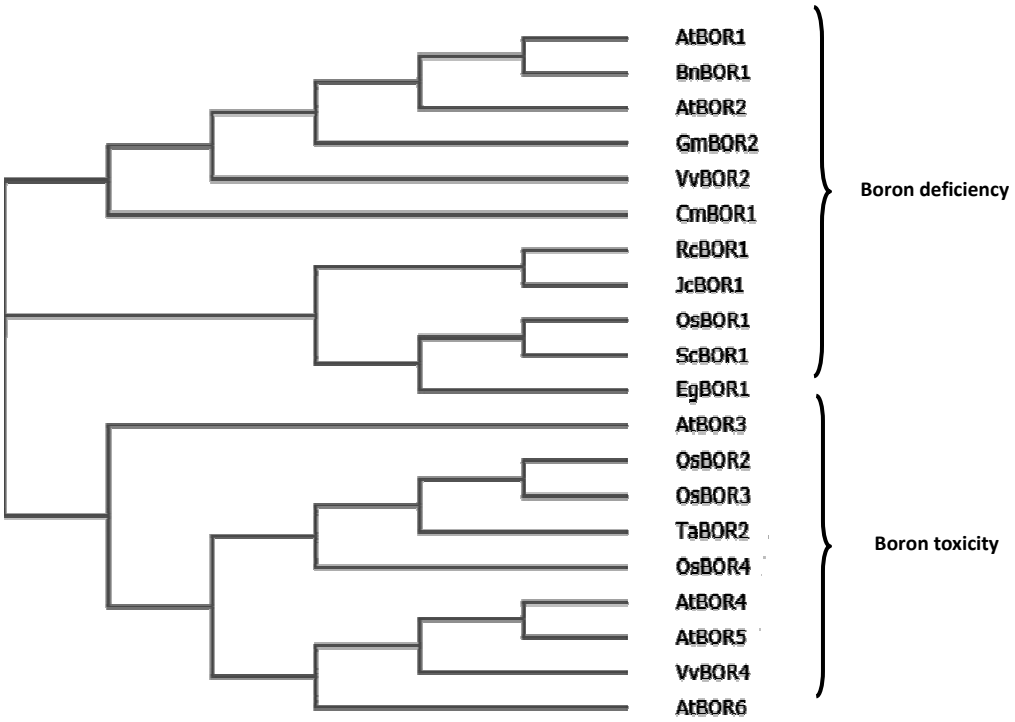

Fig. 3. The phylogenic relationship between amino acid sequence of EgBOR1 (oil palm), ScBOR1 (sugarcane) and JcBOR1 (physic nut) and amino acid sequence of BORs of other plant species. The protein accession numbers : AtBOR1(NP_850469.1), AtBOR2 (NP_191786.1), AtBOR3(NP_187296.2), AtBOR4 (NP_172999.1), AtBOR5 (NP_177619.2), AtBOR6 (NP_197925.4), TaBOR2 (ABX26206.1), VvBOR2 (XP_002272575.1), VvBOR4(XP_002281778.2), RcBOR1(XP_002519293.1), GmBOR2 (XP_003554567.1), CmBOR1 (ABQ52428.1), OsBOR1 (AAQ02664.1), OsBOR2 (ABD78950.1), OsBOR3 (EEE53967.1), OsBOR4 (ABD78951.1), and BnBOR1 (ADF30179.1)

All genes in this group are up-regulated under boron limitation and essential for efficient xylem loading of boron (Takano et al. 2002). It means that EgBOR1, ScBOR1 and JcBOR1 are important for efficient transport of boron across the plasma membrane under boron limitation (Miwa and Fujiwara, 2010). The other group is tolerance to high boron condition. The genes in this group are *AtBOR3*, *OsBOR2*, *OsBOR3*, *TaBOR2*, *OsBOR4*, *AtBOR 4*, *AtBOR5*, *VvBOR4* and *AtBOR6*. Under normal or high boron supply, BOR1 protein is transferred from the plasma membrane via the

endosomes to the vacuole for degradation. These results establish that endocytosis and degradation of BOR1 are regulated by boron availability, to avoid accumulation of toxic levels of boron in shoots under high boron supply, while protecting the shoot from boron deficiency under boron limitation (Takano et al. 2005).

Expression of *BOR1* cDNA in roots and leaves of oil palm, sugarcane and physic nut

The transcription levels of *BOR1* cDNA were determined in oil palm, sugarcane and physic nut grown under boron deficiency using real-time PCR. RNA was isolated from roots and leaves at 7, 14 and 21 days of boron deficiency. The results showed that the transcription level of *BOR1* was highest in root at 21 days in all plant species. The transcription levels of *BOR1* cDNA in root at 21 days of boron deficiency were about 3 times higher than those of the leaves (Fig. 4).

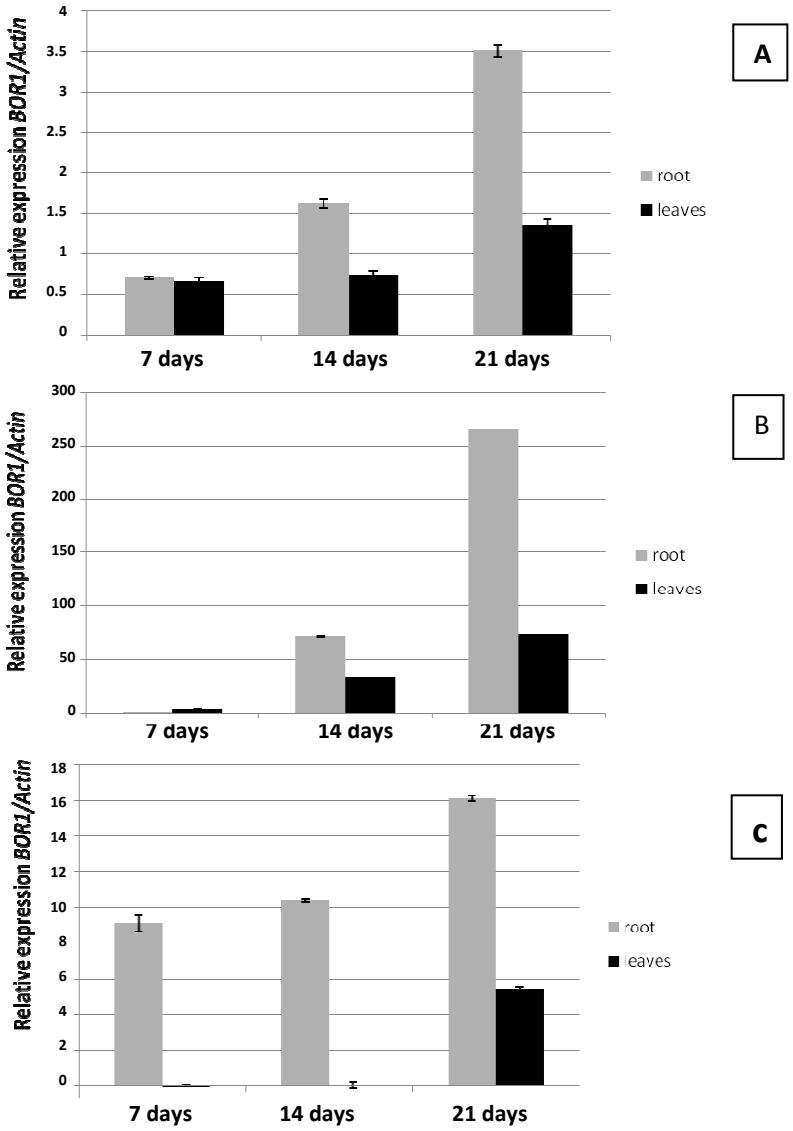

Fig. 4. *BOR1* expression level in root and leaves of plants subjected to boron deficiency for 7, 14 and 21 days. *BOR1* expression in root and leaves of oil palm (A) sugarcane (B) and physic nut (C)

This result corresponds with *BnBOR1* (*Brassica napus*) that expresses strongly in root (Sun et al. 2012) and also *Arabidopsis*, *AtBOR1* that is mainly express in root (Takano et al. 2002). The expression of *BOR1* cDNA in roots is higher than in leaves maybe because this element in these plants is immobile. In boron deficiency condition, root has to uptake boron from soil solution and export the element to all organs. In such condition plant responds to boron deficiency by increasing the expression of the gene in root for transporting more boron to other organs in plant. Thus, *BOR1* expression is higher in root than in leaves when boron concentration in soil is low or in plant become low.

CONCLUSION

Cloning of partial *BOR1* cDNA of oil palm, sugarcane and physic nut using RT-PCR of cDNA template with primers specific for *BOR1* cDNA was performed and yielded 298 bp (oil palm), 293 bp (sugarcane) and 311 bp (physic nut) of DNA fragment. The sequence analysis and comparison to the database revealed that the DNA fragment derived from oil palm, sugarcane and physicnut had similarity to *BOR1* of castor bean (83%), *Fragaria vesca* (85%), soybean (83%), grape (80%) and *Brassica napus* (78%). This infers that the synthesized cDNA is a part of *BOR1* cDNA of these plant species. The analysis of *BOR1* cDNA expression of three plant species by real-time PCR showed that the *BOR1* cDNA was expressed in roots at higher level than that in leaves when the plants were subjected to boron deficiency condition. In the future we will amplify the full length of *BOR1* cDNA of all three plant species and characterize the gene for the final goal to develop a specific marker for selecting oil palm, sugarcane and physic nut that has tolerance to boron deficiency.

ACKNOWLEDGEMENT

This study was supported by Higher Educational Strategic Scholarships for Frontier Research Networks of Thailand Commission on Higher Education and Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education. (AG-BIO/PERDO-CHE) and the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education commission, Ministry of Education, Thailand.

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