การโคลนและศึกษาลักษณะของยีนออสโมตินในเปลือกลำต้นของยางพารา ที่เกิดโรคเปลือกแห้งแบบมีอาการ Necrosis

Cloning and Characterization of an *osmotin* gene in the Bark of Rubber Tree (*Hevea brasiliensis*) Suffering with Trunk Phloem Necrosis

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บทคัดย่อ

ยางพาราเป็นพืชผลิตน้ำยางที่มักประสบปัญหาการเกิดโรคเปลือกแห้งแบบมีอาการ necrosis ซึ่งทำให้ผลผลิตน้ำยางลดลงอย่างมาก สาเหตุของการเกิดโรคนั้นยังไม่เป็นที่ทราบแน่ชัด อย่างไรก็ตามมีงานวิจัยที่แสดงให้เห็นว่าโรคเปลือกแห้งแบบมีอาการ necrosis นั้นอาจมีความสัมพันธ์กับสภาวะเครียดจากการขาดน้ำหรือสภาวะออสโมติก เพื่อที่จะตรวจสอบสมมติฐานนี้ จึงได้ทำการศึกษายีนออสโมตินซึ่งเป็นยีนที่มีความสัมพันธ์กับสภาวะเครียดจากการศึกษาพบว่า ยีนออสโมตินมีการแสดงออกเพิ่มขึ้นในต้นยางพาราที่เป็นโรคจากส่วนล่างของลำต้นถึงใต้รอยกรีดซึ่งเป็นบริเวณที่ปรากฏอาการ necrosis นอกจากนี้การแสดงออกของยีนออสโมตินยังถูกกระตุ้นได้ด้วยกรดซาลิไซลิก full-length cDNA ของยีนออสโมตินประกอบไปด้วย 738 นิวคลีโอไทด์ แปลเป็นกรดอะมิโน 246 ตัว โดยพบเปปไทด์ส่งสัญญาณที่ควบคุมการขนส่งโปรตีน การแสดงออกของยีนออสโมตินจากการทดลองนี้สนับสนุนสมมติฐานที่ว่าสภาวะเครียดจากการขาดน้ำอาจเป็นสาเหตุหนึ่งของการเกิดโรคเปลือกแห้งแบบมีอาการ necrosis

คำสำคัญ : ยางพารา โรคเปลือกแห้งแบบมีอาการ necrosis สภาวะขาดน้ำ สภาวะออสโมติก ออสโมติน

ABSTRACT

Rubber tree, an important plant producing natural rubber, is facing a severe disease called Trunk Phloem Necrosis (TPN) which greatly decreases the latex yield production. The cause of the TPN disease is now still unclear. However, some studies showed that it may be linked to water/osmotic stress. In an attempt to support this hypothesis, the expression of *osmotin*, a gene related to water/osmotic stress, has been investigated in the inner bark of the TPN-affected trees compared to the healthy ones. The expression of *osmotin* was up-regulated in the TPN-affected trees with continuous increasing gradient from the bottom part of the trunk to the tapping cut upon extension of the necrotic symptom. The full-length cDNA of *Hevea* osmotin cloned in this study contained an open reading frame of 738 bp encoding 246 amino acids. The N-terminal signal peptide indicated the

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secretory transport of the protein. All of these results supported the hypothesis that the occurrence of TPN disease may be linked to the water/osmotic stress.

Keywords : Rubber tree, Trunk Phloem Necrosis, Water stress, Osmotic stress, Osmotin

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INTRODUCTION

Rubber tree (*Hevea brasiliensis*) is the only commercial source of natural rubber, *cis*-1,4-polyisoprene, which is a raw material for many industries. Natural rubber comes from latex synthesized in the highly specialized cells called laticifers which are located in the inner phloem tissue of rubber tree. Rubber tree is frequently affected by a bark necrosis syndrome called Trunk Phloem Necrosis (TPN) leading to a sharp decrease in latex production and substantial economic losses. In general, TPN is characterized by a necrotic patch (brownish) at the inner phloem starting at the bottom part of the trunk, almost always nearby the rootstock-scion junction, and then spreading upward to the tapping cut and sometimes even above. The occurrence of necrosis in the internal phloem and the latex vessels causes the cessation of latex production (Nandris *et al.*, 1991). The trees cannot recover their latex production even after resting (stop tapping) for several years. TPN has also been observed even in some few untapped trees and no evidence of pathogen transmission could be proven (Pellegrin *et al.*, 2007). Up to now, the cause of the TPN disease still remains unclear.

A set of genes differentially expressed in healthy and TPN trees were identified by suppression subtractive hybridization (SSH) technique (Kongsawadworakul *et al.*, 2006). Among them, a number of genes involved in the water and osmotic stresses including *osmotin* was found in TPN specific SSH library. Moreover, the studies of leaf water potential, stem water potential, and pre-dawn base potential revealed that the TPN-affected trees exhibited higher water potential than the healthy ones. This indicated that TPN-affected trees may have faced some water stresses (Nandris *et al.*, 2004a, b). Therefore, the expression of *osmotin* gene in relation to the onset of the TPN was further investigated.

Osmotin is a kind of plant pathogenesis-related (PR) protein belonging to the PR-5 family. Its accumulation could be observed under both bitoic and abiotic stresses (Zhu *et al.*, 1995). Osmotin proteins tend to resist to proteolysis, pH and heat-induced denaturation which may be due to the presence of high cysteine contents (Hoffmann-Sommergruber, 2000). *Osmotin* gene expression was induced in response to salicylic acid indicating its possible role in plant defense response (Kim *et al.*, 2002)

In this study, a full-length cDNA encoding a *Hevea osmotin* was cloned and characterized in response to the TPN disease. It was up-regulated in the TPN-affected trees when compared to healthy ones. The relationship of *osmotin* expression and water/osmotic stresses was discussed.

MATERIALS AND METHODS

Plant materials and field experiments

Bark samples from TPN and healthy trees were collected from PB260 and RRIM600 clones. For PB260 clone, the TPN-affected trees showed the symptom of internal necrosis reaching the tapping cut and exhibiting external necrotic symptoms. The samples were collected along the trunk of the TPN-affected tree: at the rootstock, at the rootstock/scion junction where the external necrotic symptom occurred, beneath the tapping cut where the internal symptoms of TPN were extending, and above the tapping cut where the tissues remained healthy. The control healthy tree samples were collected at the same positions as for the TPN-affected ones.

For RRIM600 clone, the trees exhibited late TPN symptom, i.e. bark cracking and/or peeling. The trees were left untapped for several years. This allowed the regeneration of new phloemic tissues in the TPN-affected trees. The samples were collected at 20 cm above the rootstock/scion junction. The control healthy tree samples were also collected at the same position.

For salicylic acid treatment, four batches of three homogeneous (girth) tapped PB217 trees were set up: one as control and three as the treatment replicates. The trees were treated on a 1 cm large suber slightly scrapped band, just below the tapping cut, with 1 g of palm oil emulsion containing 0.05% Tween 20 and 70 mM salicylic acid for 4, 8 and 16 hours before tapping. The control tapped tree was treated the same way with 1 g of palm oil emulsion containing only 0.05% Tween 20. The inner bark samples were collected at 5 cm below the treated area.

Total RNA extraction

Total RNA was extracted from inner bark tissues of rubber tree from the materials explained above according to the method described by Tungngoen *et al.* (2009).

Expression analysis by real-time PCR

Real-time PCR was performed as described by Tungngoen *et al.* (2009) with *osmotin* specific primers (OSM-F: 5'-GCCCTTTTCTTCACTTCCTC-3' and OSM-R: 5'-GTTTGTCCGTCCCCAGATAC-3'). The internal control genes were *elongation factor I* or *actin*. The Ct (cycle threshold) value was the cycle number of which the amplified product accumulated to yield a detectable fluorescence signal at the beginning of exponential step of PCR amplification. To compare between the TPN-affected trees and healthy trees, the relative expression was calculated as $2^{-\Delta_{Ct}}$ where $\Delta Ct = Ct_{osmotin} - Ct_{internal\ control}$. To check the effect of hormone stimulation, the formula $2^{-\Delta\Delta_{Ct}}$ was used where $\Delta\Delta Ct = (Ct_{osmotin/stimulated} - Ct_{internal\ control}) - (Ct_{osmotin/control} - Ct_{internal\ control})$. Standard error (SE) calculation and ANOVA were used for statistical and significance analyses, respectively, taking into account all three-by-three replicates.

Molecular cloning of *osmotin* full-length cDNA

The *Hevea osmotin* full-length cDNA was obtained by screening of our specific bark cDNA library constructed in ZAP Express® vector (Stratagene). DIG probe labeling was performed using PCR DIG Probe Synthesis Kit (Roche). The positive clones were then excised from the phagemid and sequenced.

RESULTS AND DISCUSSION

Expression analysis of *osmotin* gene in the TPN-affected trees

The expression of *osmotin* in the TPN-affected trees was investigated by real-time PCR analysis. In PB260 clone, the samples were collected at different areas of the trunk corresponding to healthy areas (rootstock and above the tapping cut), necrotic area and partially dry area between the rootstock junction and the tapping cut, respectively. The results showed that, in healthy as well as in necrotic tapped trees, this *osmotin* gene exhibited an increasing expression gradient along the bottom part of the trunk up to the tapping cut: low at the rootstock, increasing from the junction to nearby the tapping cut and decreasing again above the tapping cut (Figure 1). This indicated that in healthy trees, tapping alone, which results in latex (# 60% water) flow out leading to water loss and circulation/re-equilibration in the whole bottom part of the trunk (Lustinec *et al*., 1968), may induce water/osmotic stress. Such stress may result in a consecutive *osmotin* up-regulation in the whole inner bark beneath the tapping cut, maximum nearby the cut and decreasing in the remote areas below. The *osmotin* gene expression was systematically higher expressed at the different sampling levels in the inner bark of the necrotic trees compared to healthy trees, even though the necrotic trees gave very little latex (Figure 1). This higher expression was highly significant at the level of the necrotic area, above the rootstock/scion junction, only. This result suggested that TPN may be linked to a local water/osmotic stress in the inner bark at the bottom of the trunk. This *osmotin* gene up-regulation was also found in the late stage of TPN in trees of RRIM 600 clone of which their new phloemic tissues were regenerated, after tapping was stopped for several years (Figure 2).

Osmotin belongs to pathogenesis-related protein class 5 (PR-5) and is well known for its role in plant protection from pathogen infection. In some plants, the *osmotin* gene was expressed in response to both biotic and abiotic stresses (Zhu *et al*., 1995). Moreover, it was reported that *osmotin* gene expression and protein accumulation were elicited in response to high osmotic stress, as well as severe water stress (Singh *et al*., 1985; LaRosa *et al*., 1992; Grillo *et al*., 1995). Tapping alone is proposed to induce a local water stress at least at the level of the inner bark tissues below the tapping cut, decreasing in the remote area. Concerning the TPN disease, eco-physiological studies revealed that the TPN-affected trees exhibited higher water potential than the healthy ones. This indicated that TPN-

affected trees faced some water stresses (Nandris *et al*., 2004a, b). The higher expression of this *osmotin* gene in TPN-affected trees, especially above the rootstock/trunk junction, where the disease always start from, supported the hypothesis that TPN symptoms may be induced by the accumulation of stresses, including or resulting in a bark local water/osmotic stress.

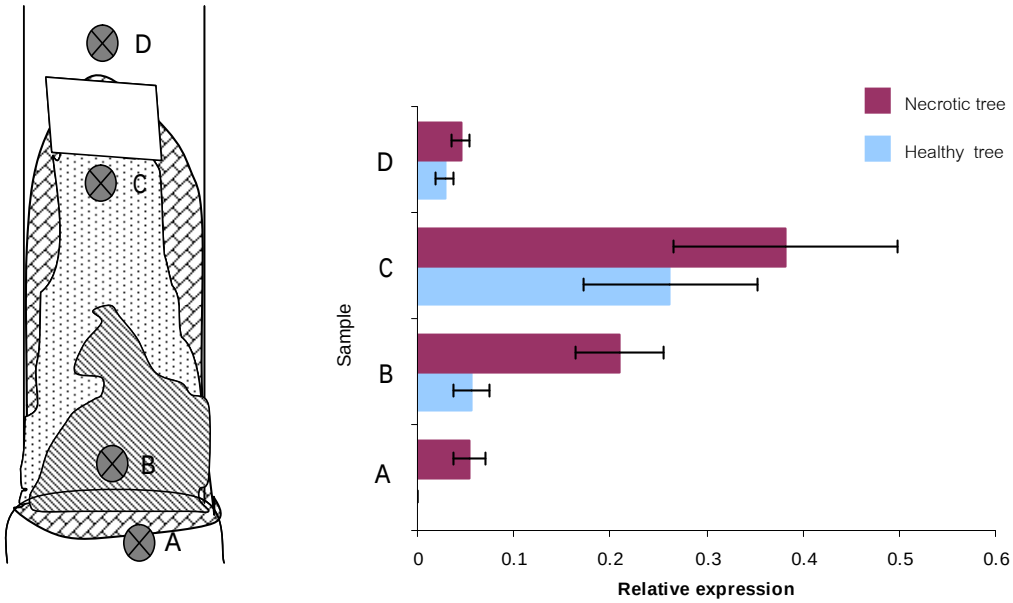

Figure 1. Real-time PCR analysis of *osmotin* gene expression in bark of healthy and TPN trees of the PB260 clone collected from the rootstock (A), external necrotic area (B), partially dry area (C) and healthy area (D). The relative expression was represented as the ratio of the transcript abundance of *osmotin* gene/transcript abundance of the control *elongation factor I* gene. Data corresponded to the mean of three independent replicates and SE (n = 9).

Expression analysis of *osmotin* gene in the salicylic acid (SA) treatment

Salicylic acid (SA) has been demonstrated to be an important signal molecule in plants' local response to pathogens and in the development of systemic acquired resistance. Moreover, SA is an important signal molecule modulating plant responses to abiotic stress including water stress (Singh and Usha, 2003). In this study, exogenous SA significantly induced the expression of this *Hevea osmotin* gene (Figure 3). The up-regulation level reached approximately three folds within 16 hours. This result suggested that SA was an effective inducer of the *osmotin* gene expression.

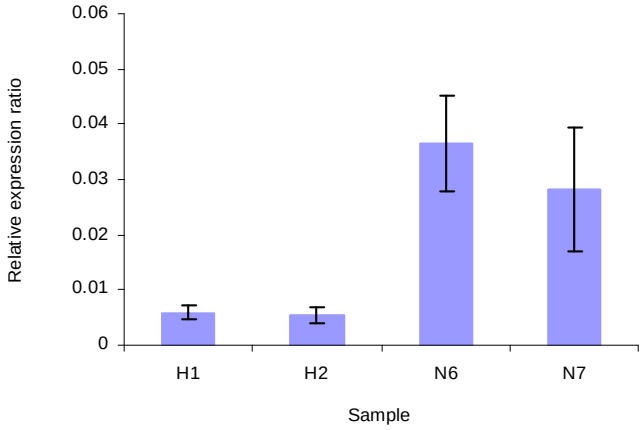

Figure 2. Relative expression ratio of the transcript abundance of *osmotin* gene/*elongation factor I* control gene in bark of healthy and TPN trees of RRIM600 clone. Data corresponded to the mean of three independent replicates and SE (n = 9). H1,H2: Healthy trees; N6,N7: TPN-affected trees.

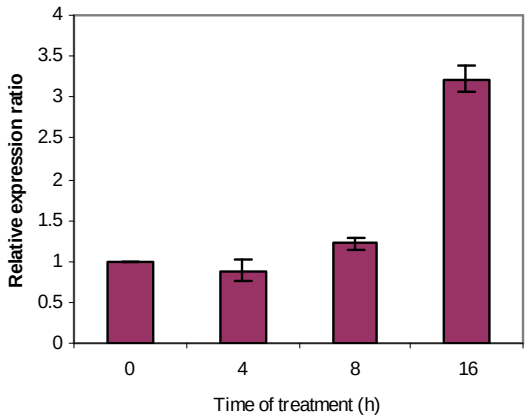

Figure 3. Relative expression ratio of the transcript abundance of *osmotin* gene/*actin* control gene in the inner bark of mature tapped trees (PB217 clone) in response to bark treatment with salicylic acid.

Cloning of the full-length cDNA encoding osmotin

The full-length cDNA of this *osmotin* (*HbOsm1*) gene contained an open reading frame of 738 bp encoding 246 amino acid preprotein. Its N-terminal signal peptide of 24 amino acids was predicted to be involved in secretory transport (Figure 4). The predicted pI value of the preprotein was 5.25 indicating that *HbOsm1* was an acidic PR-5 protein. Moreover, *HbOsm1* gene expression could be induced by SA (Figure 3) which was consistent with the tobacco acidic PR-5 gene that was also induced by SA (Niki *et al*., 1998).

Figure 4. Alignment of the deduced amino acid sequence of *Hevea brasiliensis* osmotin (*HbOSM1*) with the homologous proteins from *Gossypium hirsutum* (*GhOLP*) and *Linum usipatissimum* (*LuOLP*). N-terminal sequence was predicted as a signal peptide responsible for secretory transport.

This study provides insight into the expression of an *osmotin* gene in relation with TPN disease in rubber tree. To elucidate if the expression of this gene may be directly induced by SA and other signaling molecules, its promoter will be isolated in further experiment to determine whether it harbors a specific salicylic acid and other signals responsive *cis*-elements.

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