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Establishment of *Hevea brasiliensis* lines overexpressing genes involved in ethylene signalling pathway

Perakitan tanaman *Hevea brasiliensis* transgenik melalui overekspresi gen yang terlibat dalam jalur sinyal etilen

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Abstrak

Gas etilen merupakan hormon pada tumbuhan yang telah banyak digunakan dalam pengembangan pertanian dan hortikultura. Sebagian besar kerja beberapa gen yang responsif terhadap hormon etilen dikendalikan oleh faktor transkripsi yang disebut Ethylene Response Factors (ERF). *Dua faktor transkripsi pada tanaman* Hevea HbERF-IXc4 dan HbERF-IXc5 *merupakan gen yang ortholog dengan* ERF1 *pada* Arabidopsis thaliana. *ERF1 merupakan faktor yang mengatur integrasi sinyal antara etilen dan jasmonat. Kedua gen tersebut diduga berperan dalam pengaturan metabolisme sel lateks pada proses penyadapan dan stimulasi ethephon. Tanaman transgenik* Hevea *yang mengandung dua gen* HbERF-IXc4 *dan* HbERF-IXc5 *di bawah kendali promoter* 35S CaMV *dan* HEV2.1 *telah berhasil dirakit menggunakan metode transformasi genetik melalui* Agrobacterium tumefaciens. *Transformasi genetik melalui perantara* A. tumefaciens *dikembangkan menggunakan material kalus remah dari klon PB260. Agregat kalus* Hevea *tersebut kemudian disubkultur ke dalam medium yang mengandung paromomisin. Kalus transgenik yang diperoleh menunjukkan hasil yang positif dalam uji aktivitas GFP dengan metode Southern* blot. *Sembilan galur transgenik dikonfirmasi telah terinsersi gen* HbERF-IXc4 *dan* HbERF-IXc5, *dimana tujuh di antaranya hanya mengandung satu kopi T-DNA. Regenerasi dan karakterisasi dari tanaman transgenik perlu dilakukan untuk memahami fungsi* HbERF-IXc4 *dan* HbERF-IXc5 *yang diduga berperan dalam produksi lateks*.

[*Kata kunci: etilen*, ethylene response factor, *embriogenesis somatik, karet, faktor transkripsi, tanaman transgenik]*

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Abstract

The gaseous plant hormone ethylene has a wide variety of applications in agriculture and horticulture. Ethylene Response Factors (ERF) are the last transcription factors of the ethylene signalling pathway and control a large number of ethylene-responsive genes. Two *Hevea brasiliensis ERF, HbERF-IXc4* and *HbERF-IXc5*, are orthologs to *ERF1,* a key regulator at the crosstalk of ethylene and jasmonate signalling pathways. These genes were suggested to play an important role in regulating latex cell metabolism in response to tapping and ethephon stimulation. In this study, transgenic lines overexpressing *HbERF-IXc4* and *HbERF-IXc5* under control of *35S CaMV* and *HEV2.1* promoter have been conducted. Transgenic *Hevea* lines were obtained by *Agrobacterium tumefaciens*-mediated genetic transformation. The somatic embryogenesis process was affected by these modifications. *Agrobacterium tumefaciens* genetic transformation procedure has been developed from friable callus line for clone PB260. *Hevea* callus was sub-cultured as small aggregates on paromomycin selection medium. Transgenic callus lines established from sub-aggregates showing full GFP activity. The insertion of *HbERF-IXc4* and *HbERF-IXc5* genes were confirmed by Southern blot hybridization in nine transgenic lines, and seven of these lines have only one T-DNA copy. Further plant regeneration and characterization were necessary to understand the function *HbERF-IXc4* and *HbERF-IXc5* in latex production.

[Keywords: ethylene, ethylene response factor, somatic embryogenesis, rubber, transcription factor, transgenic lines]

Introduction

Hevea brasiliensis (Willd. ex A. Juss.) Müll Arg. is the source of natural rubber (NR), which is synthesized in latex cells. NR is an

important industrial material for transportation, consumer, and medical. The demand for NR is increasing from year to year. Nowadays, about 93% of NR world supplies is produced in Asia, Africa (4%), and Latin America (3%). The largest NR producing countries are Thailand (38%), Indonesia (30%), and Vietnam (9%). NR is the main product from *H. brasiliensis* which is obtained from latex in laticifer after tapping the bark.

Ethylene is an important hormone to stimulate latex production in rubber tree (Clément-Demange *et al.,* 2007; Putranto *et al.,* 2015; Vaysse *et al.,* 2012). The ethylene signalling pathway activates the Ethylene Response Factor (ERF) transcription factors. *ERF* genes are associated with response to abiotic and biotic stresses. *ERF1* was suggested to be a key component for the defence responses through the integration of ethylene (ET) and jasmonic acid (JA) signalling pathways (Benavente & Alonso, 2006; Lorenzo *et al.,* 2003; Wasternack & Hause, 2013). The crosstalk between ET and JA signalling pathways acts together synergistically during plant defence against herbivores, necrotrophic fungi infections, plant pathogens, and in responses of wounding (Lorenzo *et al.,* 2003; Zhu & Lee, 2015). Crosstalk connection between pathways in plants could be interpreted as the interaction between specific signal transduction pathways which resulted in a rapid and efficient mechanism for optimizing non-cognitive behaviour in response to various combinations of stimuli (Weiss and Ori, 2007).

Several genes in *H. brasiliensis* were regulated independently in signalling pathways. Defence responses in *Hevea* were dramatically mediated by wounding, methyl jasmonate (MeJA), and ethylene (ET) (Duan *et al.,* 2010). Two *Hevea ERF*, *HbERF-IXc4* and *HbERF-IXc5*, are orthologs to ERF1 a key regulator at the crosstalk of ethylene and jasmonate signalling pathways (Duan *et al.,* 2013; Piyatrakul *et al.,* 2014; Putranto *et al.,* 2015). Functional analysis of a candidate gene, *HbCuZnSOD*, was carried out in *Hevea* (Leclercq *et al.,* 2012), with both somatic embryogenesis and *A. tumefaciens*-mediated genetic transformation procedures (Blanc *et al.,* 2006; Leclercq *et al.,* 2010). One of commonly used promoters is the *35S* RNA promoter sequence from the Cauliflower Mosaic Virus (*CaMV*) (Wu *et al.,* 2014). In the other hand, promoter of the *HEV2.1* gene was reported to drive expression in latex and leaves (Montoro *et al.,* 2008). This paper aims to establish *H. brasiliensis* transgenic lines for the two recently identified transcription factors HbERF-IXc4 and HbERF-IXc5 under the control of *35S CaMV* or latex-specific *HEV2.1* promoter.

Materials and methods

The construct used for this transformation experiment consisted of a binary pCamway 2300 vector with the cassette harbouring *GFP* gene, *NPTII* gene, and one candidate gene (Leclercq *et al.,* 2015). In this study, *HbERF-IXc4* and *HbERF-IXc5* genes were cloned under the control of *35S CaMV* or *HEV2.1* promoter (Duan *et al.,* 2013; Leclercq *et al.,* 2010; Montoro *et al.,* 2008). The binary vectors were introduced into *A. tumefaciens* strain EHA105 by electroporation. Plant material used in this study was the friable callus line CI07060 from clone PB 260, which was established from integument calli (Lardet *et al.,* 2009). This callus line was sub-cultured every two weeks on a maintenance culture medium (MM). After getting the transgenic cell, the process was continued by using the green fluorescent protein (GFP) selection as a visual marker (Leclercq *et al.,* 2010). The GFP visualisation was performed under a fluorescent stereo microscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany). The production of embryos and their conversion into plantlets were carried out as described by Lardet *et al.* (2007). The development of pro-embryos was then carried out in a temporary immersion system (RITA®, CIRAD, Montpellier, France). For plant regeneration, well-shaped mature embryos were collected and transferred to DEV3 medium for germination (Lardet *et al.,* 2007).

Southern-blot hybridization analysis were conducted to verify the insertion and overexpression of two candidate genes (*HbERF-IXc4* and *HbERF-IXc5*) into *Hevea* transgenic plants genome. DNA extraction from leaves of transgenic lines was conducted as described in Leclercq *et al.* (2010). Samples of genomic DNA were fragmented with *Eco*R1 restriction enzyme and fractionated by electrophoresis in a 0.8% agarose gel in TAE buffer. The hybridization was performed as described in Sambrook *et al.* (1989) using random primed ^{32}P radio-labelled probes corresponding to *NPTII* gene (Amersham™ Megaprime DNA Labelling System, Buckinghamshire, UK).

Results and Discussion

Hevea callus was successfully sub-cultured as small aggregates on paromomycin selection medium (Figure 1A). Somatic embryogenesis was initiated for 4 weeks by sub-culturing 1 g of callus showing full GFP activity on semi-solid embryogenesis expression medium (EXP). All GFP-positive lines were obtained from DM medium then continued to transfer on EXP medium (Figure 1B and 1C). Pro-embryo development was carried out in a temporary immersion system (RITA®, CIRAD, Montpellier)

for 4 weeks with 1 min of immersion per day in the liquid development medium (DEV) (Figure 1D). Each RITA was considered as an experimental replication. Conversion of mature embryos was carried out according to Lardet *et al.* (1999). Well-shaped mature embryos were produced (Figure 1E). Plantlets were successfully derived from normal embryos (Figure 1F).

Twenty-nine GFP-positive lines were established and cryopreserved for *35S*::*HbERF-IXc4* (5 lines), *HEV2.1::HbERF-IXc4* (6 lines), *35S*::*HbERF-IXc5* (7 lines) and *HEV2.1::HbERF-IXc5* (11 lines) (Table 1). Plantlets were regenerated for wild-type and 12 of the 29 GFP-positive lines. Regeneration efficiency (number of plantlets/RITA) is lower for transgenic plants compared to wild-type, and it was very low for lines harbouring *35S*::*HbERF-IXc4*. Southern-blot molecular hybridization was performed using *NPTII* gene probe and DNA samples from plants of 9 GFP-positive lines and one negative control (wild-type) (Table 2). All these transgenic lines have 1 copy of the T-DNA except transgenic lines TS18A69 and TS18A09, which have 2 and 3 copies, respectively.

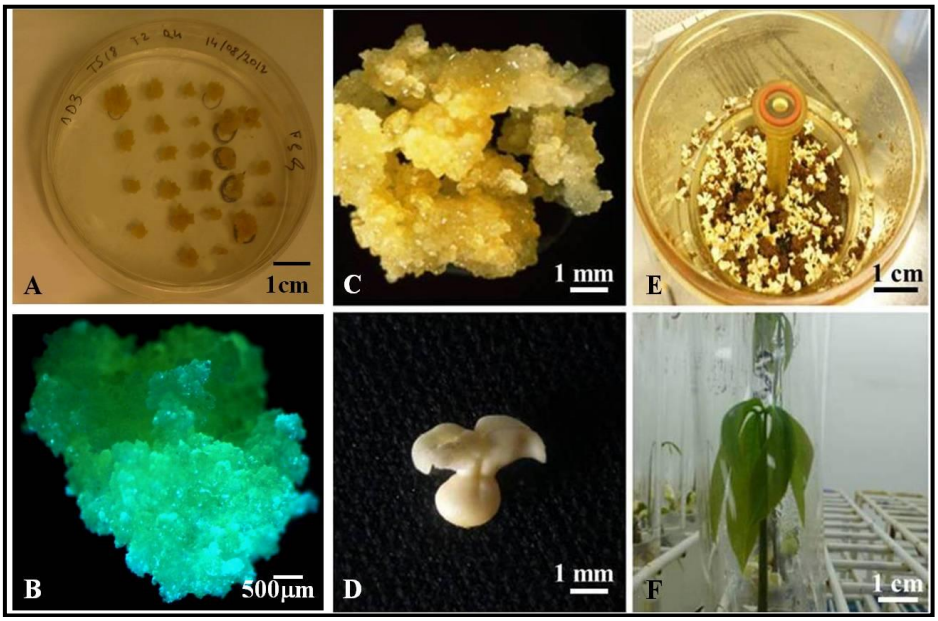

Figure 1. Somatic embryogenesis of *H. brasiliensis.* (A) paromomycin-resistant calli with GFP positive aggregates, (B) GFP fluorescence in callus, (C) Transgenic callus line, (D) Embryos from transgenic callus line, (E) Well-shaped mature embryo, (F) Transgenic plantlet.

Gambar 1. Embriogenesis somatik tanaman H. brasiliensis. *(A) Kalus GFP positif resistan terhadap paromomycin, (B) GFP fluoresens pada kalus, (C) Kalus transgenik, (D) Embrio dari kalus transgenik, (E) Embrio dengan morfologi baik, (F) Planlet transgenik.*

Table 1. Number of GFP-positive lines and their regeneration capacity.

Tabel 1. Jumlah transgen yang positif terhadap penanda GFP dan kemampuan regenerasinya.

Candidate gene *Gen kandidat*	GFP-positive lines (No) *Galur GFP positif (No)*	Line regenerating plantlets (No) *Planlet hasil regenerasi (No)*	Plantlet/RITA (No) *Planlet/RITA (No)*
None	0	1	11.94
35S::*HbERF-IXc4*	5	2	0.12
HEV2.1::HbERF-IXc4	6	5	9.44
35S::*HbERF-IXc5*	7	2	7.53
HEV2.1::HbERF-IXc5	11	3	7.82

Table 2. Southern-blot molecular hybridization analysis of DNA from wild-type (CI07060) and transgenic lines using *NPTII* probe.

Tabel 2. Analisis molekular DNA dari wild-type (CI07060) dan tanaman transgenik dengan metode Southern-blot hibridisasi dengan NPTII probe.

Construct/ *Konstruk*	Line/ *Galur*	T-DNA(No of copy)/ *T-DNA (Jumlah kopi)*
Wild-type	CI07060	0
35S::*HbERF-IXc4*	TS17A61	1
HEV2.1::HbERFIXc4	TS18A09	3
	TS18A13	1
	TS18A37	1
	TS18A69	2
35S::*HbERFIXc5*	TS19A46	1
	TS19A90	1
HEV2.1::HbERF-IXc5	TS20A69	1
	TS20A75	1

In this study, transgenic lines harbouring two genes (*HbERF-IXc4* and *HbERF- IXc5*) were successfully transformed under control of *35S CaMV* and *HEV2.1* promoters. This experiment used four constructs described as: *35S*::*HbERF-IXc4*, *HEV2.1::HbERF-IXc4*, *35S*::*HbERF-IXc5*, and *HEV2.1::HbERF-IXc5*. The transgenic lines under the construct *35S::HbERF-IXc4* showed low plant regeneration. This suggests that *HbERF-IXc4* has a negative effect on plant regeneration when driven by *35S CaMV*.

The role of gene regulatory regions (promoters) is important for understanding the regulation of plant gene expression. Both of promoters have been demonstrated to drive a candidate gene in genetic transformation via *A. tumefaciens* (Blanc *et al.* 2006; Montoro *et al.* 2000; Rattana *et al.* 2001). The *35S CaMV* promoter allows strong constitutive expression in all tissues, in the other hand *HEV2.1* promoter allows targeted expression in laticifer cells and in leaves (Montoro *et al.*, 2008).

Twenty-nine GFP-positive lines were established on paromomycin selection medium. Paromomycin is more efficient than kanamycin for the selection of transformed cells and can inhibit the growth of non-transformed cells more quickly (Montoro *et al.*, 2003; Perez-Barranco *et al.*, 2009). Nine lines were confirmed by Southern blot hybridization and seven of these lines have only one T-DNA copy. This revealed that the genetic modification methodology did not affect too much the *Hevea* genome compared to other procedures using particle bombardment or too virulent *Agrobacterium* strains. For that reason, most of transgenic lines can be compared to each other without any strong effect of the copy number. Each line with one copy of T-DNA had a higher gene expression of their corresponding transgene.

HbERF-IXc4 gene having lower callus proliferation was observed with the *35S CaMV* promoter compared to *HEV2.1*. For instance, *HbERF-IXc4* gene with *35S CaMV* need more subcultures than with *HEV2.1* to get a sufficient quantity of callus for further plant regeneration and callus cryopreservation. All lines could produce abundant yellow callus. At the beginning of culture, the callus appeared creamy in colour and then gradually became yellow or dark yellow. Finer (1988) has been classified the cotyledon callus based on the colour as green, yellow, white, brown, and red. Only yellow callus could produce embryonic culture (Finer, 1988; Perera and Dahanayake 2015; Sen *et al.*, 2014). Callus turned brown at the advance of embryo formation. Brown calli produced a large number of somatic embryos compared to yellow calli. The abnormal types of embryos cannot develop into plantlet. Some studies verified that the constitutive promoter *35S CaMV* is a weak promoter for transgene expression in young olive somatic embryos. This promoter was more active in an organized tissue of mature alfalfa somatic embryos than in the less-organised tissues of young embryos (Perez-Barranco *et al.* 2009; Tian *et al.* 2000).

The number of total embryos was similar for the two promoters (*35S CaMV* and *HEV2.1*). The rate of conversion of embryos into plantlets was lower for *35S CaMV* than *HEV2.1*. Finally, that led to produce a low quantity of plantlets for lines harbouring *35S*::*HbERF-IXc4* compared to lines harbouring *HEV2.1*::*HbERF-IXc4*.

Transgenic lines had lower plant regeneration efficiency than wild-type, and lines harbouring *ERF* genes under the control of the *HEV2.1* promoter provided a larger number of plantlets/RITA compared with those under the control of *35S CaMV* promoter especially for *HbERF-IXc4* gene. This observation suggests that

constitutive expression of *ERF* transgenes decreased regeneration efficiency. By contrast, the use of specific promoter had less effect on somatic embryogenesis process except a stronger callus browning during embryo induction.

Conclusions

This work described the successful establishment of transgenic lines carrying the two transcription factors HbERF-IXc4 and HbERF-IXc5 under the control of *35S CaMV* or *HEV2.1* promoters. Transgenic lines obtained may lead to better understanding the functional analysis of overexpression of *HbERF-IXc4* and *HbERF-IXc5* by further application of biotic and abiotic stresses and histology analysis.

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