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Overexpression of *EcGSH1* induces glutathione production and alters somatic embryogenesis and plant development in *Hevea brasiliensis*

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A B S T R A C T

Oxidative stress occurring during *in-vitro* culture is detrimental for clonal propagation by somatic embryogenesis in particular in *Hevea brasiliensis*. In previous work, overexpression of the gene encoding the cytosolic reactive oxygen species detoxification enzyme *HbCuZnSOD* led to reduce somatic embryo regeneration in *Hevea*. In this study, the role of antioxidant was tested by overexpression of the *E.coli GSH1* gene involved in glutathione biosynthesis in rubber embryogenic callus lines. Transgenic lines were successfully established and some plants were regenerated. Overexpression of *EcGSH1* gene led to glutathione over-accumulation, and affected dramatically the somatic embryogenesis process and plant development. Upon a water deficit treatment, these plants displayed the greatest drop in photosynthetic nitrogen use efficiency, higher proline content, and higher glutathione reductase activity. Changes induced in transgenic lines overexpressing *HbCuZnSOD* and *EcGSH1* were discussed as well as possible applications on plant material propagation, overcoming loss of natural rubber production through Tapping Panel Dryness, and tropical soil remediation.

1. Introduction

Reactive oxygen species (ROS), like the superoxide ion, hydrogen peroxide and the hydroxyl radical, were long suggested to be involved in recalcitrance to tissue culture in some species, and consequently were detrimental for the clonal propagation (Benson, 2000). Oxidative stress enhances somatic embryogenesis in many plant species (Ganesan and Jayabalan, 2004; Luo et al., 2001; Pasternak et al., 2002). Indeed, slight oxidative stress is necessary to induce the somatic embryogenesis process but excessive oxidant compounds trigger the oxidation of phenols, leading to callus browning and tissue senescence. Involvement of the redox status in embryogenesis was demonstrated (Jo et al., 2014; Pan et al., 2009; Stasolla, 2010; Wickramasuriya and Dunwell, 2015). The mastery of somatic embryogenesis by controlling the redox status with exogenous antioxidants has been described in both angiosperms and gymnosperms (Becker et al., 2014; Belmonte and Stasolla, 2007; Fraga et al., 2016; Pullman et al., 2015; Vieira Ldo et al., 2012). Engineering plants with high antioxidant capacity were described in the literature (for a review Noctor et al., 1998) but the effect on somatic embryogenesis was not studied except in *Hevea*. In this plant species, oxidative stress dramatically hampers proliferation and plant regeneration (Montoro et al., 2012) with polyphenol oxidation leading to tissue browning (El Hadrami and D'auzac, 1992; El Hadrami et al., 1993; Chanwun et al., 1993). Temporary immersion system is used for *Hevea* plant regeneration by somatic embryogenesis (Etienne et al., 1997). This system induces a substantial oxidative stress, which is revealed by a higher superoxide dismutase activity and lipid peroxidation (Martre et al., 2001). Effective reduction of tissue browning could be obtained by supplementing culture media with polyamine, silver nitrate or activated charcoal (Auboiron et al., 1990; Carron et al., 1995; El Hadrami and D'auzac, 1992) (Carron et al., 1992), as well as growth regulators, which reactivate embryogenic cells (El Hadrami and D'auzac, 1992; Montoro et al., 1992). Transgenic *Hevea* plants overexpressing the mitochondrial *HbMnSOD* gene (Jayashree et al., 2003) and the cytosolique isoforme *HbCuZnSOD* have been regenerated successfully, in absence of a post-transcriptional regulation of *HbCuZnSOD* transcript by miR398 in *Hevea* (Gébelin et al., 2012; Leclercq et al., 2012). Both somatic embryogenesis and plant growth were affected in transgenic lines. The low rate of plant regeneration by somatic embryogenesis in *HbCuZnSOD* transgenic lines was related to the absence of callus browning usually observed in non-transformed

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lines. Two *HbCuZnSOD* lines produced fast-growing and very vigorous plants (Leclercq et al., 2012).

Glutathione (GSH), a tripeptide of glutamic acid, cysteine and glycine (Hopkins, 1929), plays a central role in ROS detoxification system, which protects cells from damage caused by these cytotoxic molecules (for review (Noctor and Foyer, 1998; Scandalios, 2005). The glutathione biosynthesis pathway takes place thanks to 2 ATP-dependent enzymes: γ–glutamate cysteine ligase (γ-ECS, EC: 6.3.2.2) and glutathione synthetase (GSH-S, EC: 6.3.2.3). γ-ECS combines glutamate and cysteine to give a dipeptide, γ-L glutamyl-L-cysteine (γ-EC), to which a glycine residue is added to the C-terminal to form glutathione (Rennenberg, 1982). The limiting factors in this reaction are cysteine concentration and γ-ECS enzyme activity (Noctor et al., 2011). Numerous feedbacks in the biosynthesis pathway have been shown in animals and plants (Rennenberg, 1982) (Alscher, 1989). These feedbacks can be circumvented by using bacterial genes (Watanabe et al., 1986). Over-expression of the GSH-S bacterial gene in poplar did not lead to the over-accumulation of GSH (Strohm et al., 1995; Herschbach et al., 1998 #74). However, over-expression of the γ–glutamate cysteine ligase gene of *E. coli* (γ-ECS; EcGSH1) has been successfully used in *Populus x Canescens* and Populus *tremula x* Populus *alba* poplar trees, (Arisi et al., 2000; Gullner et al., 2001; Gyulai et al., 2014; Gyulai et al., 2005; He et al., 2015; Herschbach et al., 2010; Ivanova et al., 2009; Ivanova et al., 2011).

This study aims to analyse the effect of an increase in glutathione on somatic embryogenesis by overexpressing the *E. coli GSH1*. This gene was introduced into *Hevea* under the control of the *CaMV 35S* and *HbHEV2.1* promoters (Montoro et al., 2008). The *CaMV 35S* promoter enables strong constitutive expression in all tissues, whereas the *HbHEV2.1* promoter enables targeting transgene expression in laticifer cells and in leaves (Montoro et al., 2008). Genetic modification of the rubber tree is routinely carried out from embryogenic callus (Blanc et al., 2006; Montoro et al., 2003; Montoro et al., 2000; Rattana et al., 2001) and transformation events are selected using the *green fluorescent protein* (*GFP*) reporter gene (Leclercq et al., 2010). Over-expression of the *EcGSH1* transgene led to glutathione over-accumulation but had some dramatic effects on the somatic embryogenesis process and on plant development.

2. Materials and methods

2.1. Plant materials

The friable callus line CI05519 of *Hevea* clone PB260 was established as decribed in (Lardet et al., 2009). Prior to *Agrobacterium* inoculation, the callus line was precultured for 15 days in glass tubes on preculture medium (PM), namely a $CaCl_2$-free MM medium supplemented with 1.35 μM BAP and 3,4-D (Montoro et al., 2003).

2.2. Binary vectors and Agrobacterium strain

The two binary vectors had a pCAMBIA 2300 backbone, a pPZP-based small binary vector (Hajdukiewicz et al., 1994), with the *neomycin phosphotransferase II* (*NPTII)* gene conferring resistance to neomycin under the CaMV35S promoter. The first binary vector (pCAMBIA2300-GFP-EcGSH1) was constructed with the *GFP* S65T reporter gene containing a StLS1 intron 2 from pCAMBIA30063 (Vancanneyt et al., 1990) and a modified *EcGSH* gene ORF (gi: 41622; (Watanabe et al., 1986)) where the starting codon TTG was replaced by ATG, under the CaMV35S promoter or *HEV2.1* promoter (Montoro et al., 2008). The binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA 105 by electroporation. The bacteria inoculum was prepared as described in (Montoro et al., 2000; Rattana et al., 2001).

2.3. Inoculation and selection of transgenic calli

Inoculation was performed as described previously (Blanc et al., 2006). To isolate transgenic callus lines, GFP-positive aggregates were successively subcultured every 3 weeks on decontamination medium (DM) and then several times on DM with increasing concentrations of paromomycin from 50 to 150 mgL^{-1} (Rattana et al., 2001). Finally, transgenic callus lines were established from sub-aggregates showing full GFP activity (Leclercq et al., 2010; Leclercq et al., 2012). GFP visualisation was performed on callus at the end of each subculture under a fluorescence stereomicroscope and macroscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany) using the GFP2 filter (480 nm excitation filter/510 nm barrier filter). These callus lines were then subjected to molecular characterization, plant regeneration and/or cryopreservation according to the protocol described previously (Lardet et al., 2007). The mastering of the process allows the production of clonal plantlets for a given transgenic callus line. For traceability reason, transgenic lines are named according to the genetic transformation experiment order (TS12 is the 12th experiment done). T is for the co-culture duration [a coculture during 3 days (T1), 4 days (T2) or 5 days (T3)]. A is for the name of the GFP positive aggregate given after DM1 medium and kept all over the GFP selection process until the line establishment.

2.4. Genomic DNA extraction from callus and Southern–blot hybridization

DNA from transgenic callus lines was isolated as described in (Leclercq et al., 2012). Ten micrograms of genomic DNA was fragmented with *Eco*RI restriction enzyme and fractionated by electrophoresis in a 0.8% agarose gel in TAE 1X buffer. After transfer onto a Hybond N^+ nylon membrane (Amersham Biosciences, England), hybridization was performed as described in (Sambrook et al., 1989), using random primed ^{32}P radio-labelled probes corresponding to the *NPTII* gene amplified with the following primers: NPTII-F: 5′- CCGGC TACCTGCCCATTCGA-3′ and NPTII-R: 5′-GCGATAGAAGGCGATGCG-3′. The number of bands reflected the number of T-DNA insertions.

2.5. Plant regeneration

The production of embryos and their conversion into plantlets were carried out as described in (Lardet et al., 2007). The experiment was performed with five independent replications.

To compare the regeneration ability of wild-type and transgenic callus lines, wild-type callus line CI05519 was cultured over the length of the modification experiment and regenerated. Plant regeneration was initiated once enough transgenic callus had been produced. For both non-modified and transgenic callus lines, the number of total embryos per gram of callus (T), the number of well-shaped embryos per gram of callus (WS), the number of plantlets per gram of callus (P) and the conversion percentage (P/WS) were recorded.

2.6. RNA extraction from leaves

Four 1-year-old plants per line were used for gene expression analysis. The RNA extraction procedure used has been previously described in (Duan et al., 2010). Total RNAs were quantified with Nanoquant (Tecan, Männedorf, Switzerland) and conserved at −80 °C.

2.7. Complementary DNA (cDNA) synthesis

Before cDNA synthesis, the absence of contaminating genomic DNA was checked on all RNA samples by performing a PCR reaction with *HbActin* primers HbActin-F: 5′-AGTGTGATGTGGATATCAGG-3′, HbActin-R: 5′-GGGATGCAAGGATAGATC-3′(Duan et al., 2010). In case genomic DNA had been detected, a DNAse treatment was performed using TurboDNAse (Ambion, Texas, USA) following the manufacturer's instructions. Four micrograms of DNA-free RNAs was used for cDNA in