Transgenic plants over-expressing *HbCuZnSOD* cytosolic isoform are more tolerant to a water deficit

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Abstract

Reactive oxygen species (ROS) scavenging systems are involved in various biotic and abiotic stresses. Genetic engineering of Hevea was attempted to strengthen plant defences against ROS accumulation. *Hevea* transgenic plant lines over-expressing cytosolic *HbCuZnSO*D gene driven by a 35S CAMV promoter were successfully established and regenerated. The over-expression affected somatic embryogenesis and in plant development. The water deficit tolerance of two fast-growing *HbCuZnSOD* over-expressing lines (TS5T3Af and TS4T8An) was evaluated. Plants from line TS4T8An displayed lower stomatal conductance, a higher proline content and an activation of all ROS-scavenging enzymes suggesting a better protection against ROS.

Introduction

ROS are generated by biotic and abiotic stresses. The antioxidant metabolism protects cells from oxidative damage caused by ROS. In plants, several enzymes act jointly to maintain redox homeostasis. ROS scavenging systems play an important role in cell functioning because ROS are extremely cytotoxic. Many antioxidant enzymes catalyse redox reactions, such as the ascorbate-glutathione cycle. Of the antioxidant enzymes, superoxide dismutase (SOD) activity constitutes the first line of defence against ROS by converting $O_2^{\cdot -}$ to H_2O_2. Transgenic plants over-expressing the mitochondrial *HbMnSOD* gene have been regenerated, but further characterization under stress conditions has yet to be reported (Jayashree *et al.*, 2003). We studied the effect of over-expression of the cytosolic *HbCuZnSOD* gene in *Hevea brasiliensis*. Transgenic plants were regenerated using the GFP-based transformation procedure (Leclercq *et al.*, 2010). Successful *Hevea* genetic transformation of the homologous *HbCuZnSOD* gene led to the generation of transgenic lines. Over-expression affected somatic embryogenesis and plant development. Two transgenic lines regenerated fast-growing plantlets, one of them displaying greater osmoprotection with reduced stomatal conductance and better ROS protection, with higher antioxidant enzyme activities under a water deficit.

Results and discussion

Production of the HbCuZnSOD *transgenic callus lines*

Seventy-two 35S::SOD transgenic callus lines were established using GFP selection. Southern-blot hybridizations were carried out on fully fluorescent GFP callus lines. Sixty-two lines harboured a single copy of T-DNA, six lines had two copies and four lines had truncated insertions. The relative transcript abundance of the *HbCuZnSOD* gene was attempted by real-time RT-PCR on twelve 35S*::GUS-35S::GFP* control lines (Leclercq *et al.* 2010) and forty-nine *35S::HbCuZnSOD* lines harbouring a single copy of T-DNA.

Plant regeneration from HbCuZnSOD *transgenic callus lines*

Plant regeneration was carried out on one non-transformed control line (CI05519), four transformed control GFP/GUS lines (TS3T4Ab, TS3T4Ac, TS3T4A22, TS3T4A24) and twenty *HbCuZnSOD* transformed lines (TS4 and TS5) with one single copy of T-DNA. The highest number of total and well-shaped embryos, and plantlets per gram of fresh matter of callus was recorded for the non-transformed CI05519 lines. However, these best well-shaped embryo and plant production rates in the non-transformed control were not significantly different with those obtained for two transformed control lines (TS3T4Ac and TS3T4A22) and six *HbCuZnSOD* transformed lines (TS4T2A23, TS4T2A44, TS4T8Ah, TS4T8Aj, TS4T8An and TS5T3Af). As usual, all callus lines regenerating embryos and plantlets turned brown after embryogenesis induction. In contrast, some *HbCuZnSOD* transgenic callus lines remained yellow and proliferating. These lines were affected at various steps of the embryogenesis process: three lines did not produce any embryos, seven lines did not produce any well-shaped embryos and eleven lines did not produce any plantlets. In our case, proliferation of callus in the RITA® system without necrosis suggested better ROS detoxification and maintenance of a reduced environment throughout the somatic embryogenesis process, which is putatively deleterious for embryo formation.

Survival rate and plant growth in HbCuZnSOD *over-expressing lines*

Although no obvious morphological difference was observed between the wild-type, the transgenic control lines and the *HbCuZnSOD* over-expressing lines, survival dramatically decreased in most *HbCuZnSOD* lines. After acclimatization, the survival rate of the transgenic control plants from TS3 lines was over 90% after 2 and 6 months. After 1 year, the survival rate of those transgenic control lines decreased to reach a minimum of 82%. In contrast, the survival rate of *HbCuZnSOD* over-expressing lines was lower after 2 months with a minimum of 75%, and then decreased again to 33.3% after 6 months and one year. However, no significant difference was observed, except for lines TS4T8Af and TS4T8Ah, 12 months after acclimatization. At the same time as the survival rate was measured, stem heights were recorded at the acclimatization stage and after 12 months. At the beginning of acclimatization, no significant difference was seen between the wild-type, the transgenic control lines and the *HbCuZnSOD* over-expressing lines. After one year of acclimatization, plants from line TS4T8An and TS5T3Af were significantly taller. Further physiological and biochemical characterizations were conducted on these two fast-growing *HbCuZnSOD* over-expressing lines.

Biochemical and physiological responses to a water deficit treatement

Plants from the two *HbCuZnSOD* lines TS5T3Af and TS4T8An and the transgenic control line TS3T4Ab were dehydrated at various FTSW from 1 to 0.1. After twenty-one days without watering, the transformed control and TS5T3Af plants displayed leaf symptoms associated with a water deficit: leaf curling followed by leaf yellowing, highlighting the hydathodes. In contrast, plants from line TS4T8An were still healthy. The proline content significantly increased under the water deficit. However, a discrepancy was observed between the plant lines. Line TS4T8An displayed the highest level of proline compared to the transformed control line and the other *CuZnSOD* line TS5T3Af. Several physiological parameters, such as stomatal conductance (g_s), the time needed to reach FTSW = 0.1 and the performance index (PI_{abs}), were monitored as the FTSW was decreased. In fact, without any treatment, the initial stomatal conductance of the TS4T8An plants was significantly lower than in the plants from the TS5T3Af and TS3T4Ab control lines. The time needed to reach a FTSW of 0.1 distinguished between the TS4T8An plants and the TS5T8Af and control plants. Plants from line TS4T8An took 25 days without any watering to reach a FTSW of 0.1,

compared to the 14 days taken by the other two plant lines, probably due a lower transpiration rate in line TS4T8An. Further physiological analyses are needed for a better understanding of the role of antioxidant systems in line TS4T8An against oxidative stress. Moreover, TS4T8An plants displayed reduced stomatal conductance. Saving more water meant that it took 25 days to reach a FTSW of 0.1 when 17 days were necessary for the other lines. The high rate of growth under normal conditions and the low water consumption under water stress suggest the plants of this line have better water use efficiency.

Conclusion

Targeting *HbCuZnSOD* in the cytosol is promising as the oxidative burst generated by a strong water deficit treatment revealed the tolerance potential of plantlets from line TS4T8An, such as a higher proline content, lower stomatal conductance and activation of all ROS-scavenging enzymes. The over-expression of *HbCuZnSOD* cytosolic isoform could improve the ROS scavenging system against an oxidative burst whatever its origin. Targeting transgene expression specifically in latex cells could help to improve latex redox homeostasis for better protection of lutoids against oxidative damage, by using the promoter from gene *HEV2.1*, which encodes the major latex Hevein protein (Montoro *et al.*, 2008).

Acknowledgements

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References

Jayashree R, Rekha K, Venkatachalam P, Uratsu SL, Dandekar AM, Kumari Jayasree P, Kala RG, Priya P, Sushma Kumari S, Sobha S, Ashokan MP, Sethuraj MR and Thulaseedharan A (2003) Genetic transformation and regeneration of rubber tree (Hevea brasiliensis Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Plant Cell Reports* **22**:201-209.

Leclercq J, Lardet L, Martin F, Chapuset T, Oliver G and Montoro P (2010) The green fluorescent protein as an efficient selection marker for Agrobacterium tumefaciens-mediated transformation in Hevea brasiliensis (Mull. Arg). *Plant Cell Rep* **29**:513-522.

Montoro P, Lagier S, Baptiste C, Marteaux B, Pujade-Renaud V, Leclercq J and Alemanno L (2008) Expression of the *HEV2.1* gene promoter in transgenic *Hevea brasiliensis*. *Plant cell, tissue and organ culture* **94**:55-63.

Program
2011 IRRDB International Rubber Conference
14 - 17 December 2011 in Chiang Mai Thailand

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14 December 2011

14.00 – 17.00 **: Registration**

15 December 2011

Room 1

08.00 – 08.45 **: Registration**

09.00 – 09.50 **: Opening Plenary**

- Welcome Address by Vice Governor of Chiang Mai Province, Mr.Woragan Yokying
- Statement by the Secretary General of the IRRDB
- Opening Speech by Deputy Director – General, Department of Agriculture, Ms.Mantana Milne
- Presentation of the IRRDB B.C. Sekhar Award for Research Excellence
- Citation to be read by Mr. Hubert Omont

09.50 – 10.20 **: Coffee break**

Chairman: Mr. Hubert Omont, CIRAD

10.20 – 10.45 **: Invited paper 1** Facing Challenges in Rubber Diseases (Dr. C.K. Jayasinghe)

10.45 – 11.10 **: Invited paper 2** Progress on Rubber Breeding of Thailand (Dr.Napawen Lekawipat)

11.10 – 11.35 **: Invited paper 3** How Rubber will Bounce in a Volatil World (Dr. Stephen Evans, Secretary General, IRSG)

11.35 – 12.00 **: Invited paper 4** Factor Determing NR Prices in the Short and Medium Term (Jom Jacob, Senior Economic, ANRPC)

12.00 – 13.00 **: Lunch**

Exploitation And Physiology

Chairman: Dr. James Jacob, Director RRI India,

13.00 – 13.15 **: Paper 1** Low Frequency Tapping Systems as for the Solution of Labour Crisis (Panus Paechana)

13.15 – 13.30 **: Paper 2** Influence of Tapping System on Rubber clone ; RRIT 251 (Sajeerat Raemlee)

13.30 – 13.45 **: Paper 3** Assembly of gss-cut Exploitation System on Slow Starter Clones and Stimulantion With Ethylene GAS (Atminingsih)

13.45 -14.00 : **Paper 4** The "Double Cut Alternative" (DCA) Tapping System : An Innovative Tapping System Designed for Thai Rubber Smallholdings Using High Tapping Frequency.
(Eric Gohet)

14.00 – 14.15 : **Paper 5** Carbohydrate Variation of Hevea Induced by Tapping Systems
(Pisamai Chantuma)

14.15 – 14.30 : **Paper 6** Effects of Drought and Tapping for Latex Production on Water Relations of Hevea Brasiliensis Trees
(Mr.Sumit Kunjet)

14.30 – 14.45 : **Paper 7** A Modified Stimulation Method in *Hevea* Brasiliensis for Reducing Oxidative Stress
(R. Krishnakumar)

14.45 – 15.00 : **Paper 8** Sudden Dieback of Young Budded Rubber (*Hevea brasiliensis* Muell. Arg.) Plants at Nursery Stage Under Hot and Dry Climatic Conditions
(A. M. W. K. Senevirathna)

15.00 – 15.30 : **Coffee break**

Chairman: Dr. Chairil Anwar, Director Indonesian RRI

15.30 - 15.45 : **Paper 9** Wood Production and Lumber Recovery of 4 Recommended Rubber Clones in Thailand
(Krissada Sangsing)

15.45 – 16.00 : **Paper 10** Analysis of Wood Quality in Hevea Drasiliensis : Estimation and Quantification of Lignin bio-Polymer and Cell Wall Phenolics
(Dr. C.P.Reghu)

Plantation Agronomy

Chairman: Mr. Suchin Maenmeun, Director RRI Thailand, Chairman of the IRRDB

16.00 – 16.15 : **Paper 11** Model of Developing and Strengthening of Rubber Nursery Institutional to Improve the Quality of Plnting Material and Productivity of Indonesian Rubber Smallholdings
(Lina Fatayati Syarifa)

16.15 – 16.30 : **Paper 12** Modelling of Hevea Yield Production Based on Clone, Soil, And Climate Potential
(Imam Susetyo)

16.30 -16.45 : **Paper 13** Growth Characteristics of Five Rubber Clones at High Elevation Area in South Sumatra
(Thomas Wijaya)

16.45 -17.00 : **Paper 14** Growths and Carbon Stocks of Para Rubber Plantations on Phonpisai Soil Series in Northeastern Thailand
(Chakarn Saengruksawong)

18.00 : **Dinner**

15 December 2011

Room 2

Pests And Diseases Management

Chairman: Prof. Dr. Liu Guodau, Director of RRI CATAS, China

13.00 – 13.15 : **Paper 1** Corynespora Leaf Fall on Rubber in Vietnam, Current Status and Recent Studies
(Phan Thanh Dung)

13.15 – 13.30 : **Paper 2** Pathogenicity Assay of Corynespora Cassiicola Isolates From Rubber Tree and Other Hosts in Vietnam
(Nguyen Don Hieu)

13.30 – 13.45 : **Paper 3** Epidemic of *Corynespora* Leaf Fall on Rubber Orchard in Cote D'Ivoire : Assessment *in Vitro* of Clonal Resistance and Fungicides Effectiveness.
(Wahounou P. J)

13.45 -14.00 : **Paper 4** Differential Expression Analysis by CDNA-AFLP of *Hevea brasiliensis* After inoculation with the Pathogen *Corynespora Cassiicola*
(Huang Guixiu)

14.00 – 14.15 : **Paper 5** Efficacy of Fertilizers to Control White Root Disease of Rubber Caused by *Rigidoporus Microporus* at the Early Planting Stages
(Mrs.Arom Rodesuchit)

14.15 – 14.30 : **Paper 6** Study on the Potency of Serratia Bacteria Used to Control White Root Disease in Rubber
(Tri Rapani Febbiyanti)

14.30 – 14.45 : **Paper 7** The Cell Differentiation Observation at the Early Infection Stage of Oidium heveae and a Method of RNA Extraction of This Pathogenic Fungus
(Mr. Wan Sanlian)

14.45 – 15.00 : **Paper 8** Establish the Indoor Identification Model of Rubber Tree (*Heveae brasiliensis*) to Powdery Mildew (*Oidium heveae*)
(Tu Min)

15.00 – 15.30 : **Coffee break**

Chairman: Dr. Yin Song, Director Cambodian RRI

15.30 -15.45 : **Paper 9** Disease Caused by Botryodiplodia Theobromea Pat on Rubber Tree in Vietnam : Current Status and Recent Studies
(Tran Anh Pha)

15.45 – 16.00 : **Paper 10** Cultural and Morphological Characterizations of *Fusicoccum* sp., the causal agent of rubber (*Hevea brasiliensis*) leaf blight in Malaysia
(Nyaka Ngobisa Aurelie)

Plantation Agronomy

Chairman: Prof. Osayanmo Eguavoen, Director RRI Nigeria

16.00 – 16.15 : **Paper 11** Forestation With Rubber for Carbon Markets ; Yield Tables Under Srilankan Conditions
(E. S. Munasinghe)

16.15 – 16.30 : **Paper 12** Estimation of Rubber Stand Age in Typhoon and Cold Weather Afflicted Area With Landsat Tm Data : A Case Study in Hainan Island, China
(Bangqian Chen)

18.00 : **Dinner**

16 December 2011

Room 1

Breeding and Biotechnology

Chairman: Dr. Lai Van Lam, Director RRI Vietnam

08.30 – 08.45 : **Paper 1** Performance of RRIT 251 Clone in Traditional and Non-Traditional Area
(Ms.Patra Kinnaret)

08.45 – 09.00 : **Paper 2** Adaptation of Rubber Tree (*Hevea Brasiliensis*) Clones to Marginal Areas of Cote D'Ivoire
(ELABO A.A.E.)

09.00 – 09.15 : **Paper 3** *Hevea Brasiliensis* : Results From The Cambodian Large Scale Clone Trials in 2011
(Dr. Phen Phearun)

09.15 - 09.30 : **Paper 4** Potential of IRR 200 Series Promising Rubber Clones on Further Trials in Indonesia
(Aidi Daslin)

09.30 – 09.45 : **Paper 5** Performances of Elite Rriv's Clones Derived From 1994 Hand Pollination Program
(Le Mau Tuy)

09.45 -10.00 : **Paper 6** Genetic Variabilty of Interspesific Crossing Result Between RRIM 600 X PN 1546 Rubber Parental Clones
(Sekar Woelan)

10.00 – 10.30 : **Coffee break**

Chairman: Mr. N'diaye Oumar N'gor, FIRCA, Cote d'Ivoire

10.30 – 10.45 : **Paper 7** Climate Change: Study on Stability of Rubber Clones in Field Trials
(Dr. Nasaruddin Md. Aris)

10.45 – 11.00 : **Paper 8** Polycross Breeding Towards Evolving Genetically Diverse Hevea Clones for Sustainbility
(Dr. Kavitha K. Mydin)

11.00 -11.15 : **Paper 9** Attempts to Evolve Compact Crown Clones of Hevea Brasiliensis (T. Gireesh)

11.15 – 11.30 : **Paper 10** Early Performance of a Few Indigenous and Exotic Clones of *Hevea Brasiliensis* in a Large Scale Trial (V.C. Mercykutty)

11.30 – 11.45 : **Paper 11** Evaluation of Some Clones (Hevea Brasiliensis) in a Small Scale Trial in the Southern Part of Guatemala (Gremial de Huleros)

11.45 – 12.00 : **Paper 12** Application of QTL Mapping for Early Selection on Growth and Latex Yield Traits in Rubber Breeding. (Ms. Ratchanee Rattanawong)

12.00 – 12.15 : **Paper 13** Identification of Drought Tolerant Genes by Quantitative Expression Analysis in *Hevea Brasilensis* (Mohamed Sathik)

12.15 – 13.00 : **Lunch**

Chairman: Dr. Mohd Nasaruddin Md. Aris, Director of Production Development, Malaysian Rubber Board

13.00 – 13.15 : **Paper 14** Genetic Analysis and Population Structure of Rubber Tree for Association Mapping. (Dr.Thitaporn Phumichai)

13.15 – 13.30 : **Paper 15** Identification of Rubber Clones (Hevea Brasiliensis) Using Inter Simple Sequence Repeat (ISSR) Markers (Hoang Thi Lieu)

13.30 – 13.45 : **Paper 16** Development and Characterization of EST-SSR Markers From *De Novo* Transcriptome Sequencing Data in Rubber Tree (*Hevea Brasiliensis*) (Dejun Li)

13.45 – 14.00 : **Paper 17** Progress of RRIT's *Hevea* Somatic Embryogenesis (Wittaya Prommee)

14.00 – 14.15 : **Paper 18** Exploitation of in Vitro Induced Zygotic Polyembryony for Genetic Transformation in *Hevea Brasiliensis* (Rekha. K)

14.15 – 14.30 : **Paper 19** Unfertilized Ovule - A Potential Explant for Somatic Embryogenesis in *Hevea Brasilensis* (Jayashree,R.)

14.30 – 14.45 : **Paper 20** Histochemical and Immunohistochemical Identification of Laticifer Cells in Callus Cultures Derived From Anthers of *Hevea Brasiliensis* (Deguan Tan)

14.45 – 15.00 : **Paper 21** Improvement and Application of the Technique of Mini-Seedling Budding of *Hevea Brasiliensis*
(Lin Weifu)

15.00 – 15.30 : **Coffee break**

Chairman: Mr. Nicomedes Eleazar, Director BAR, Philippines

15.30 – 15.45 : **Paper 22** Identification of *HbNIN2* as the Key Invertase Responsible for Sucrose Catabolism in Rubber-Producing Laticifers, A Rate-Limiting Step Determining Rubber Productivity
(Chaorong Tang)

15.45 -16.00 : **Paper 23** Transgenic plants over-expressing *HbCuZnSOD* cytosolic isoform are more tolerant to a water deficit
(P. Montoro)

16.00 – 16.15 : **Paper 24** Addressing the fears of the natural rubber supply chain regarding the dissemination of genetically modified rubber trees
(Pascal Montoro)

Plantation Agronomy

16.15 – 16.30 : **Paper 25** Effect of Slow Release Fertilizer on Three-Whorl Polybag Rubber Planting.
(Ramli Abd Majid)

16.30 – 16.45 : **Paper 26** Establishment of Standard Values for Nutritional Diagnosis in Soil and Leaves of Immature Rubber Tree
(Mrs.Saichai Suchartgul)

16 December 2011

Room 2

End Use And Processing Technology

Chairman: Dr. R.B. Prenadasa, Director Ganeral, Rubber Development Department, Sri Lanka

08.30 – 08.45 : **Paper 1** Solar Drying Chamber with Furnace for Rubber Sheet
(Mrs.Preprame Tassanakul)

08.45 – 09.00 : **Paper 2** Biomolecules Contents and Rubber Properties of Some *Hevea Brasiliensis* Clones
(Dr.Chatchamon Daengkanit Nathaworn)

09.00 – 09.15 : **Paper 3** The Latest Development in the Application of the Seismic Rubber Bearing Technology
(Kamarudin Ab Malek & Le Jiang Jun)

09.15 - 09.30 : **Paper 4** The Design of Compound Formulae Based on Natural Rubber Blend for Engine Mounting Preparation
(Dadi R. Maspanger)

09.30 – 09.45 : **Paper 5** Porous Pipe Production for Agriculture
(Ms.Sumana Jammeuan)

09.45 -10.00 : **Paper 6** A Method for Producing Carbon Black Silica Master Batch
(Rosamma Alex)

10.00 – 10.30 : **Coffee break**

Chairman: Dr. Jerome Saint Beauve, CIRAD

10.30 – 10.45 : **Paper 7** Utilization of waste fo Rubber Plantation and Crumb Rubber Factory as a Source of Bioenergy.
(Didin Suwardin)

10.45 -11.00 : **Paper 8** SWIM-BED Reactor for the Treatment of Latex Concentrate Wastewater.
(Nguyen Ngoc Bich)

11.00 – 11.15 : **Paper 9** Synthesis of Silvernanoparticles Using *Hevea* Leaf and Latex
(Jayasree Gopalakrishnan)

Social - Economic

Chairman: Dr. Stephen Evans, Secretary General IRSG

11.15 – 11.30 : **Paper 10** The Development of Forward Market Model
(Ms.Athiwee Daengkanit)

11.30 – 11.45 : **Paper 11** Future Prospect for Udon Thani as an SEZ for Rubber Industry
(Suthee Intraskul)

11.45 – 13.00 : **Lunch**

Chairman: Dr. Karyudi, Head Sungei Putih Research Centre, Indonesian RRI

13.00 – 13.15 : **Paper 12** Performance Evaluation of Rubber, Hevea Brasiliensis, Towards Commercialization in Quezon Province.
(Dr. Cecilia N. Gascon)

13.15 – 13.30 : **Paper 13** Nong Khai Rubber Learning Centre Oriented by Famers' Participation
(Mr.Kaset Nabsanit)

13.30 – 13.45 : **Paper 14** Integrated Farming in Rubber Plantation Along Chai Pattana Foundation
(Mr. Thongchai Kamkote)

13.45 – 14.00 : **Paper 15** Transfer of Technology Project in Davao Region, Philippines.
(Alfredo Cayabyab)

14.00 – 14.15 : **Paper 16** Community-Based Participatory Action Research (CPAR) on Rubber-Based Farming System in the Philippines.
(Roger O. Bagaforo)

14.15 – 14.30 : **Paper 17** Grassroots Level Rubber-Based Agroforestry Initiatives in the Philippines: Capitalizing On Lessons and Experiences
(ROSELYN F. DAELMO (UPLB))

14.30 – 14.45 : **Paper 18** Anticipated Constraints for Sustainable Smallholder Rubber Farming: A Case Study in the Moneragala District of Srilanka.
(Mrs.Wasana Wijesuriya)

14.45 - 15.00 : **Paper 19** JDM88 Agro-Ventures Rubber Based Farming System (Jerry Gil S. Murao)

15.00 – 15.15 : **Paper 20** Rubber- Based Agroforestry in Five States : Effort Towards Conserving Farmers' Priotized Medicinal Plants in Nigeria. (Mrs. Eseosa S. Osazuwa)

17 December 2011

09.00 -17.00 : **Field Trip to The International Horticulture Exposition Royal Flora Ratchapruek 2011**