Towards a latex molecular diagnostic of yield potential and the genetic engineering of the rubber tree

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Abstract

The latex from *Hevea* is a rubber producing cytoplasm expelled from specialized cells upon bark tapping. The rubber yield is mainly limited by the duration of the latex flow, which is controled by coagulation processes. Bark treatment with ethylene is known to delay coagulation and increase latex yield.

The molecular basis of latex coagulation has been characterized:

1) Hevein, a lectin-like protein, induces latex coagulation by bringing together the rubber particles (RPs). The hevein-RPs bridging is mediated by N-Acetyl-D-glucosamine, and involves a 22 kDa receptor glycoprotein localized on the RPs surface. This process is inhibited by the removal of the sugar moiety from the receptor, through the action of N-acetyl-glucosaminidase and chitinases.
2) Ethylene induces, in the latex cells, an over-expression of the 3 genes: chitinase, hevein and its receptor. The higher over-expression of one chitinase can explain the partial deglycosylation of the hevein receptor and the resulting delay in coagulation.
3) The level of hevein and chitinase expression in the latex is a clonal characteristic, linked to the characteristics of the latex flow. Expression of these genes might be used as molecular markers for high yield potential.

Based on these findings, it would be interseting to genetically engineer the rubber tree to get new promising high yielding cultivars with prolonged latex flow.

Introduction : the laticifers, latex and the rubber-yield limiting factors.

The latex of *Hevea brasiliensis* is a rubber-containing cytoplasm expelled from the laticifers, upon bark wounding or deliberate tapping. The laticifers (de Faÿ & Jacob, 1989) are periodically emitted from the cambium as single cells, forming "latex cells mantels" in the soft bark of the rubber tree. Upon maturation, the latex cells in each new mantel turn highly specialized in the synthesis of rubber, and fuse together, forming a network of anastomozed latex vessels, allowing their cytoplasm to flow out easily upon bark wounding.

As a true cell cytoplasm, the latex contains, suspended in the cytosol, all the organelles of non-photosynthetic plant cells (d'Auzac & Jacob, 1989) : vacuoles (the lutoids), plastids, mitochondria, nuclei and endoplasmic reticulum (de Faÿ *et al.*, 1989). It countains also polysomes (Coupé & Chrestin, 1989) and numerous rubber particles accounting for 35-45% of the total latex volume and for more than 90% of the latex dry matter.

Natural rubber from *Hevea* consists of long chains of *cis*-polyisoprene which are

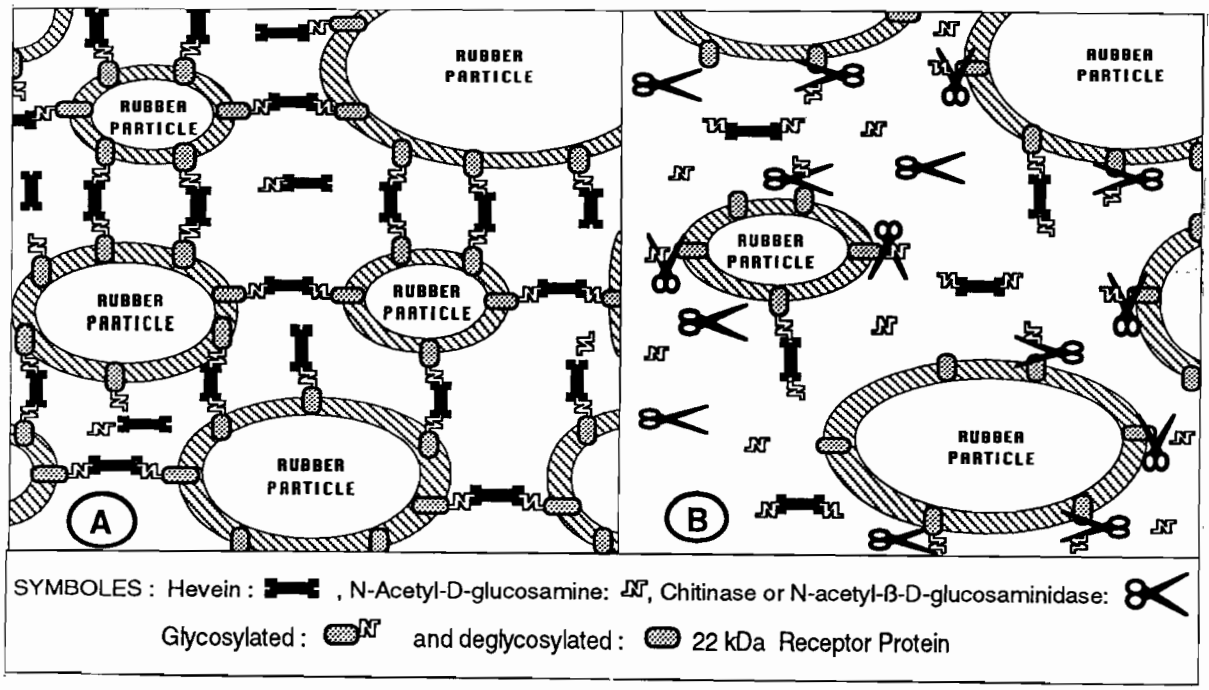

Figure 1 : Model for coagulation of latex. **A** : efficient coagulation of latex occurs because of high concentration of hevein (lectin) which can bind to the to the glycosylated moiety of the hevein receptors, on the surface of the rubber particles, bringing them together in a tridimensionnal network and resulting in their agglutination. **B** : coagulation is delayed or inhibited because of high chitinase activity which provoques deglygosylation of the hevein receptor, resulting in its inefficiency to bind hevein. Further, the resulting high concentration of free N-acetyl-D-glucosamine in the cytosol can saturate the binding sites of hevein which also become inefficient in inducing the process of coagulation

synthetized, through the mevalonate pathway (Keckwick, 1989) from acetyl-CoA derived from glycolysis. Polymerization of isopentenyl pyrophosphate by the prenyl-transferase, assisted by the Rubber Elongation Factor (REF), gives rise to the long chains of *cis*-polyisoprene, with an average molecular weight ranging from 0.7 to 40 x 10^5, that is to say isoprenic chains more than 5 x 10^3 to 3 x 10^5 carbons long. The polyisoprene chains are grouped together and form aggregates called the "rubber particles", surounded by a lipoproteic membrane.

In *Hevea* species, latex yield is a clonal character (Jacob et al. 1989). Nowadays a yield of 5,000 liters of latex - ie ≈ 2 tons of dry rubber - per year per hectar is considered as very high in the estates, while in small scale trials, with optimum tapping sytems, yield could reach up to 4 tons dry rubber/year/ha. As such, the rubber tree can be regarded as a true photo-autotrophic bioreactor, specialized in the synthesis of natural rubber.

Two intrinsic factors are known to limit the latex yield at the level of the latex producing tissues of the rubber tree :

- the duration of the latex flow, which determines the volume of latex collected at each tapping. The duration of the flow is limited by the coagulation, leading to the plugging of the extremities of the severed latex vessels (d'Auzac, 1989);

- the capability of the latex cells to regenerate the exported latex between two tappings (Jacob *et al.*, 1989);

Bark treatment with Ethephon (an ethylene releaser) is known to transiently overcome the 2 intrinsic limiting factors by delaying coagulation, and stimulating the latex metabolism (regeneration), therefore resulting in increased latex yield (Coupé & Chrestin,

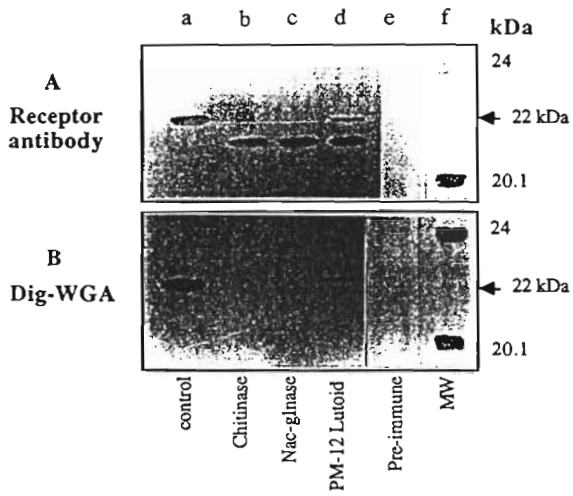

Figure 2 : Effects of various exogenous enzymes and a lutoid extract on the glycosylated status of the hevein receptor protein from the latex rubber particles (Western blotting). **A** - Detection with the antibody raised against the hevein receptor. **B** - Detection with the digoxigenin labelled wheat germ agglutinin (WGA)

a- control (crude rubber particles protein extract) ; **b**- treatement 30 min with commercial exochitinase ; **c**- treatment with commercial N-acetyl-glucosaminidase ; **d**- incubation 30 min with the lutoids sap, ultrafiltrated on PM-12 in order to eliminate hevein and retain chitinases (upper part) ; **e**- detection with preimune serum ; **f**- molecular markers

1989) . But overstimulation can induce an oxidative stress within the latex cells, leading to the "Tapping Pannel Dryness" (TPD) (Chrestin *et al.* 1984; Chrestin, 1989), a laticifer physiological desorder resulting in the definitive cessation of latex production.

All these characteristics (phenotypes : duration of the latex flow, latex regeneration, response to yield stimulation and sensitivity to TPD) have been proven to depend on clones (genotypes), hence should be genetically determined (gene expression).

Differential gene expression in *Hevea* latex cells has already been reported (Kush *et al.* 1990) as well as the increase of some genes expression in response to yield stimulation with ethylene (Broekaert *et al.*, 1990; Pujade-Renaud *et al.*, 1994). This paper essentially focuses on the expression of the genes involved in the processes of rubber coagulation in relation with rubber yield and ethylene stimulation.

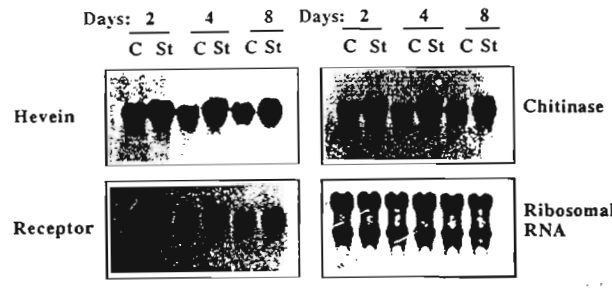

Figure 3 : Effects of a stimulation with Ethrel on the expression of hevein, the hevein receptor and chitinase genes, in the latex cells of Hevea

Northern blotting : 10 µg total RNA from latex/65°C hybridization and washings (0.5-0.1 x SSC). Ribosomal RNA stained with Methylene blue.

D-days after stimulation ; C-control ; St-stimulated trees were all tapped every 2 days. (Exposure : 10 hours)

Towards a latex molecular diagnostic of propency to coagulation

We have shown that coagulation of latex is under the control of at least 3 proteins (Gidrol *et al.*, 1994). These 3 types of proteins are hevein, a small 2-binding-sites lectin (phyto-agglutinin), a ≈ 22kDa glycosylated hevein-receptor bound to the rubber particles membrane, and chitinases. Hevein can bind to the N-acetyl-glucosamine moiety of its receptor protein and therefore can bring together the rubber particles, leading to the building of a multidimension rubber particle cohesive network and to latex coagulation (Fig. 1-a). This process can be partially - or eventually completely - inhibited by the action of endogenous chitinases, which can remove the glycosylated moiety of the hevein receptor (Fig. 2) becoming therefore ineffective in binding hevein (Fig. 1-b).

We got cDNA or genomic partial clones of these 3 genes either by PCR from the already published data on hevein (Broekaert et al., 1990) and chitinases (Jekel *et al.*, 1991; Blaiseau & Lafay, 1992) or by the immunoscreening, for the hevein receptor (Chrestin & Kush : unpublished data), of a latex specific c-DNA expression library. Using these probes, we could show that ethylene stimulation - which delays coagulation hence increases rubber yield (for more than 10 days) - paradoxically induced an overexpression of

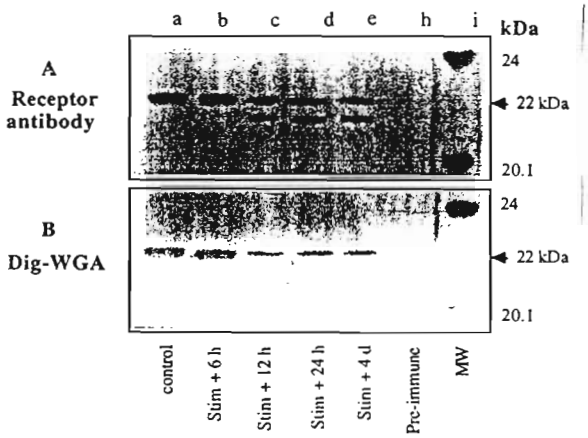

Figure 4 : Effects of bark stimulation with Ethrel (an ethylene generator) on the glycosylation of the hevein-receptor protein from the latex rubber particles (Wester blotting). **A** - Detection with the antibody raised against the hevein-receptor. **B** - Detection with the digoxigenin labelled wheat germ agglutinin (WGA)

a- control : rubber particles proteins (no treatment/1st tapping) ; **b**- 6 hours after bark stimulation with Ethrel (1st tapping) ; **c**- 12 h after the treatment (1st tapping) ; **d**- 24 hours after treatment (1st tapping) ; **e**- 4 days after the treatment (3rd tapping) ; **h**- detection with preimmune serum ; **i**- Molecular weight protein markers (Amidoblack)

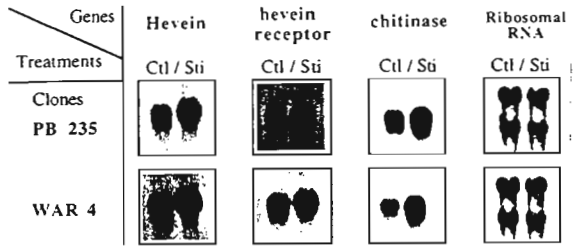

Figure 5 : Northern blot analysis of the expression of the hevein, the hevein receptor and chitinase genes in the latex cells of the rubber tree clones : PB235 (high yielding/easy latex flow) and WAR4 (poor yielding/short latex flow)

Ctl : Control not stimulated ; **Sti** : Stimulated 48 hours before tapping. All trees have been regularly tapped for at least 6 years. (Exposure : overnight)

these 3 genes involved in coagulation. But the higher overexpression of a chitinase gene in the latex cells after stimulation (Fig. 3), which led to effective partial deglyco-sylation of the hevein receptor (Fig.4) could explain the action of ethylene in delaying coagulation processes. Further the ethylene effects could last at least 8 days (Fig. 3) and other experiments could show that it could even last more than 15 days (Chrestin *et al.* unpublished data), as does the classical increase of yield after a stimulation.

Moreover, comparing 3 rubber clones with different latex flow characteristics, such as the high yielding PB-235 or GT1 genotypes (low plugging index/prolonged latex flow) and the War-4 low yielding genotype (high plugging index/short latex flow), we could demonstrate that (Fig. 5), compared with PB-235 as the GT1 genotype (Gidrol *et al.*, 1994), the latex of the War-4 genotype was characterized by a higher expression of hevein and hevein receptor (pro-coagulants) and above all by a very low constitutive expression of chitinase (anti-coagulant). Ethrel stimulation, which comparatively induced in War-4 genotype a much higher increase in the latex yield, induced a much larger increase in chitinase expression in the latex cells of War-4 genotype.

These 3 genes, and particularly hevein ("procoagulant") and chitinases genes (anti-coagulants"), the products of which compete for the same site (the N-acetyl-glucosamine moiety) of the hevein receptor, to induce or inhibit the process of coagulation, are excellent candidates as yield potential molecular markers. Such markers will be of great help in the rubber breeding programs for early selection of high yielding (good latex flow) and/or stimulation responsive rubber clones (parents and progeny).

Conclusion and prospects : Towards the genetic engineering of the rubber tree.

These results evidence that the expression level of certain genes is linked to phenotypic characteristics of the rubber trees (latex coagulability) and may be used, in the future, as tools in a latex molecular diagnostic for improvement of rubber yield. Other potential yield markers (latex regeneration, tolerance to stress) remain to be checked.

One can think that, in the future, it will be possible to succeed in the genetic engineering of the rubber tree, through the genetic transformation of embryogenic calli and regeneration then micropropagation of the

transformed trees. Using this way, it might be possible to obtain for example new *Hevea* cultivars with prolonged latex flow, or more resistant to the TPD desease. First attempts for rubber tree genetic transformation has recently been published (Arokaraj et al., 1994). Now the molecular biologists and the plant tissue culture specialists, who have been successfull in mastering the regeneration of rubber tree from somatic embryo (Montoro et al. 1993 & 1995; Carron et al. 1995), are working together to reach these objectives.

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