

Specific promoters for genetic engineering of the rubber tree; molecular and functional analysis.

Pujade-Renaud V¹, Sanier C¹, Cambillau L¹, Tharreau D¹, Puangkosol N³, Montoro P¹, Chrestin H², Narangajavana³

¹CIRAD, France

²IRD, France

³Mahidol University, Thailand

Introduction

Genetic engineering of *Hevea brasiliensis* may be one way to improve the yield of natural rubber production, by over-expressing favorable genes of the latex metabolism or by improving the defenses of the tree. It is also an interesting tool for the production of recombinant proteins of industrial interest. *Agrobacterium tumefaciens*-mediated transformation procedures allowing the genetic engineering of several *Hevea brasiliensis* cultivars are now available (Arokiaraj *et al.* 1998; Montoro *et al.* 2000, 2004; Rattana *et al.* 2001, 2004). However, the success of genetic engineering programs also relies on the use of well adapted promoters, allowing optimized expression of the transgene in the appropriate tissue, in accordance with the application foreseen. We have focused on two kinds of genes as a source of potentially interesting promoters for the control of transgene expression in rubber tree latex: hevein- and glutamine synthetase-encoding genes.

Hevein is a small lectin protein involved in latex coagulation mechanisms, and homologous to “pathogenesis related (PR)” proteins. Hevein antifungal properties were demonstrated both *in vitro* (Van Parijs *et al.*, 1991) and in transgenic plants (Lee and Raikhel, 1995). In bark tissues, it is present in the latex cells only, as shown by immunolocalization. In addition, rubber tree hevein genes are expressed at very high level and preferentially (if not specifically) in the latex compared to leaves. They are over-expressed by wounding, ethylene, and ABA (Broekaert *et al.*, 1990). They may supposedly be up-regulated by pathogen infection, like other hevein-like genes of the same family (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Potter *et al.*, 1993). The genes coding for rubber tree hevein are therefore good candidates for cloning promoters guaranteeing strong, specific, and potentially inducible expression in the laticifers.

Glutamine synthetase genes are differentially expressed ethylene-inducible genes (Pujade-Renaud *et al.*, 1997, 2000). Ethylene-inducible promoters will be useful to overexpress transgenes upon ethylene treatments, taking in consideration that “stimulation” (treatment with ethephon) is a common practice in rubber farming.

Two promoters from hevein genes (H1 and H4) and 2 promoters from glutamine synthetase genes (GS2 and GS3) were previously isolated and cloned in fusion with the *gus* reporter gene, in a pCambia-derived binary vector, in order to analyse their functionality in various cellular environments. The lengths of the subcloned promoters are listed in table 1.

Molecular cloning of promoter GS1

More recently, a third GS gene promoter was isolated, by “adaptor-anchored PCR”. It corresponds to the *gs1* cDNA isoform that had been used as a probe for the screening of a genomic library which resulted in the isolation of the first two promoter sequences, GS2 and GS3.

Gs1 diverges significantly from *gs2* and *gs3* in terms of sequence. *Gs2* and *gs3* are highly homologous in their transcribed region but they differ in the promoter region. Previous Northern blot experiments, using probes specific of the *gs1* and both *gs2/gs3* isoforms,

demonstrated that both *gs1* and *gs2/gs3* are over-expressed in response to ethephon treatment. However, *gs1* presents an over expression of larger amplitude, as it is hardly expressed in the latex of untreated rubber trees. Functionality analysis of all three GS promoters will confirm whether GS1 is more responsive to ethylene than the other two.

Table 1. Size of the isolated promoter region after cloning in the binary transformation vector.

Promoter	Length
GS1	678 pb
GS2	939 pb
GS3	762 pb
H1	299 pb
H4	1776 pb

***In silico* analysis**

A number of potential regulatory elements were identified by bioinformatic analysis of the 5 promoter sequences using the PLANT CARE database as a reference (Lescot *et al.* 2002).

Several elements necessary for the regulation by hormones involved in biotic and abiotic stress responses, such as jasmonic acid, salicylic acid, ABA or ethylene, were found in hevein promoter sequences, as well as elements specifically involved in the regulation by wounding, drought, heat shock or elicitors. These data suggest that hevein gene promoters are probably highly regulated by biotic and abiotic stresses in rubber tree. Auxin- and gibberellin-responsive elements also suggest a developmental regulation.

From the *in silico* analysis, glutamine synthetase promoter sequences GS1, GS2 and GS3 also appear highly and differentially regulated, both developmentally and by stress. It is worth noting the presence, among other elements, of 3 ethylene-responsive elements in both GS1 and GS2 sequences, but none in GS3. Respectively 1, 4 and 5 wound-responsive elements were found in GS1, GS2 and GS3.

Functionality analysis in model plants

We have started testing our constructs in two plant systems for which genetic transformation is quick and highly efficient: rice, model plant for monocotyledons, and *Arabidopsis*, model plant for dicotyledons. The advantage of such model systems for studying our promoters is, not only a faster transformation/regeneration process but also the possibility to multiply easily the transgenic events through progeny. It offers the possibility to demonstrate more rapidly certain regulation traits and to extend the range of applications to other plants.

Transgenic rice plants carrying the *gus* reporter gene under the control of promoter H1, H4, GS2 or GS3, were produced following the *Agrobacterium*-mediated procedure described by Sallaud *et al.* (2003). Only the plants carrying a single T-DNA insertion were selected for further analysis.

Transgenic *Arabidopsis* plants carrying the *gus* reporter gene under the control of promoter H4 only were obtained following the *in planta* procedure described by Clough and Bent (1998).

Hevein gene promoters (H1 and H4)

Histological and fluorimetric analysis revealed that the 1.8 kb hevein upstream sequence H4 is able to drive the expression of a transgene both in rice and *Arabidopsis*. In rice, H4-*gus* expression was observed in leaves, pollen bags, 5-10% of the pollen grains, root vessels. H4-*gus* steady state level of expression in rice leaves was high (half that of the CaMV 35S

promoter). It was significantly activated by wounding (+35%), and also by inoculation with spores of *Magnaporthe grisea*, pathogen responsible for the rice blast disease (+20%). In young T1 transgenic *Arabidopsis* seedlings, H4-*gus* was expressed in the whole plantlet. For promoter H1, no clear-cut expression profile could be evidenced, as 2 transgenic lines only, out of 7, expressed the *gus* reporter gene, at a very low level. Due to its short size (0.3 kb), promoter H1 may lack elements necessary for optimal activity or may be under the influence of the genomic environment of the T-DNA insertion.

Glutamine synthetase gene promoters (GS2 and GS3)

GS2 and GS3 promoters were both functional as promoters in rice, with an average level of expression higher for GS2 than GS3. In fact, no *gus* expression at all could be measured in all 16 unwounded plants carrying the GS3-*gus* construct. After wounding, *gus* expression was induced (by a factor 5) in 4 plants out of 16. No activation of the GS3 promoter in response to ethephon could be evidenced.

On the other hand, the GS2 promoter was activated by ethephon (+60% in average), while not activated by wounding.

Conclusion and prospects

Out of 5 promoters isolated from latex genes, 4 are functional and inducible in model plants. Promoter H4 is very promising considering its high level of expression and its regulation specificities. It may prove useful in order to overexpress transgenes in situations of biotic or abiotic stress, not only in rubber tree but also in other plants.

Now that the rubber tree transformation procedure developed at CIRAD is well mastered and offers promising perspectives (Montoro *et al.* 2004), these promoters can be analysed in their original environment. In case of promoter H4, it is now important to test its tissue specificity and specific regulation by various stress relevant to rubber tree such as tapping (wounding), ethylene treatment or pathogen infection.

The cloning of a third GS promoter (GS1) now offers the possibility to thinly analyse differential regulation of the GS family members, in particular with respect to development and ethylene, whether endogenous or exogenous, through stimulation treatments.

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