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COMPARATIVE EFFICIENCY OF DIFFERENT CONSTRUCTS FOR DOWN REGULATION OF TOBACCO CINNAMYL ALCOHOL DEHYDROGENASE

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

NABILA YAHIAOUI, CHRISTIANE MARQUE, HÉLÈNE CORBIÈRE and ALAIN MICHEL BOUDET*

UMR CNRS/UPS 5546, Centre de Biologie et Physiologie Végétales, 118, route de Narbonne, 31062 Toulouse, Cedex, France

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Key Word Index—*Nicotiana tabacum*; solanaceae; cinnamyl alcohol dehydrogenase; lignin; lignification; transgenic tobacco; RNA antisense strategy; promoter.

Abstract—A number of research groups have recently succeeded in down regulating cinnamyl alcohol dehydrogenase (CAD EC.1.1.1.195) activity through genetic engineering with the aim of modifying lignin profiles in plants. In view of the expected extension of this technology to economically important plants, we have tried to obtain additional information on the best antisense constructs for maximal CAD down regulation using tobacco (*Nicotiana tabacum* cv. Samsun) as a model system. A comparison of different constructs showed that efficiency was greater with homologous cDNAs (partial or full-length sequence) vs heterologous cDNA. In addition, constitutive promoters were, in our hands, more efficient than tissue (xylem) specific promoters. Although a strong down regulation of CAD gene expression was obtained for different transformants at the *in vitro* stage, a drop in CAD activity reduction was observed for some of them during development in the greenhouse. These different aspects are discussed in the context of future applications.

INTRODUCTION

Since the first characterization of "encrusting material" which embedded cellulose in wood and the first introduction of the term "lignin" by Schulze in 1865 [1] to identify this material, an impressive amount of data has been accumulated on the chemistry, biosynthesis, distribution and properties of lignins. Recently, the techniques of molecular biology and genetic engineering have led to progress in this area and allowed new lignins, apparently more suited to different agroindustrial uses of plant biomass to be obtained in a controlled way. Lignins indeed have a negative impact on forage digestibility [2], and in the pulp industry; wood processing for pulp and paper requires the use of costly and energy consuming treatments to separate lignins from cellulose [3].

Both lignin content and lignin monomeric composition can influence wood processing [4]. Therefore, the design of plants with a decreased lignin content and/or a modification of the lignin monomer composition in order to improve pulping characteristics and forage digestibility now represents an expanding research field and significant results have already been obtained in different plant species through the modulation of a wide range of genes encoding different enzymes involved in the phenylpropanoid pathway and in the lignin specific pathway [[5], for review].

Cinnamyl alcohol dehydrogenase (CAD EC.1.1.1.195) catalyses the last step of lignin monomer synthesis by reducing cinnamyl aldehydes into cinnamyl alcohols. The first CAD cDNA was identified in tobacco [6], and the first transgenic plants with a down regulated CAD activity were obtained using the antisense strategy in tobacco as a model plant [7]. The lignin content of the down regulated plants was unchanged, whereas the monomeric composition of the lignins was affected (decreased syringyl to guaiacyl monomer ratio). The lignins of the transgenic plants were enriched with cinnamyl aldehydes and their

*Author to whom correspondence should be addressed. Tel: (33) 05 61 55 67 53; Fax: (33) 05 61 55 62 10; e-mail: amboudet@cict.fr.

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; cOMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; GRP, Glycine Rich Protein; CaMV 35S-DE, CaMV 35S promoter with a duplicated enhancer.

extractability in mild alkali conditions increased [7, 8]. The CAD down regulated tobacco plants exhibited a red coloration of the xylem that was ascribed to the increased proportion of cinnamyl aldehydes in the lignin [9, 10]. These transgenic plants were obtained with a tobacco cDNA in antisense orientation under the control of the CaMV 35S promoter and among them, some lines exhibited a strong reduction of CAD activity (up to 7% residual CAD activity). In parallel experiments on tobacco transformation, Hibino *et al* [11] used a heterologous cDNA of *Aralia cordata* under the control of the CaMV 35S promoter and obtained moderate reductions of CAD activity (45% and 80% residual CAD activity) associated with a slightly brownish coloration of the stems and an increase in the cinnamyl aldehyde content of the lignins [9]. More recently, Baucher *et al* [12] obtained transgenic poplar trees with 30% residual CAD activity by expressing a homologous poplar CAD cDNA, in sense or antisense orientation, associated to the CaMV 35S promoter. The resulting transgenic material exhibited characteristics similar to those of CAD down regulated tobacco plants.

Studies carried out on tobacco lines with strong reduction of CAD activity and on transgenic poplar trees demonstrated that the increased alkali extractability of the modified lignins was correlated to the reduction of the kappa number, an important criterion in the pulping process (indicative of the amount of residual lignin in the pulp). Thus, the CAD antisense strategy appears extremely well adapted to obtain improved plants for the pulp industry and this technology is suitable for development into commercial applications. In order to extend it to other economically important species, often more difficult to transform, it is important to obtain additional information on the optimal conditions of the antisense RNA strategy. These include the potential efficiency of heterologous constructs (thus avoiding the need to clone homologous CAD cDNAs in given species), and the potential interest of plant tissue-specific promoters to drive the CAD antisense genes in the xylem in order to avoid potential drops in defense reactions associated to lignin production in periderm or superficial tissue. With these ideas in mind, we undertook new experiments on tobacco as a model system aiming to determine the comparative efficiency of different antisense constructs. With the exception of some experiments on the impact of antisense cDNA length on down regulation of cOMT [13], such comparative studies have not been published previously for lignification genes.

RESULTS

Antisense CAD constructs

Six different antisense constructs were tested for their efficiency on CAD activity down regulation (Fig. 1). For four of them, the antisense sequences were under the control of constitutive promoters:

—the CaMV 35S promoter was fused to a full-length (1.4 kb, construct **3P**) or to a 1 kb 5′ fragment of tobacco CAD cDNA (construct **T**)

—the CaMV 35S promoter with a duplicated enhancer (CaMV 35S-DE) was associated to a full-length tobacco CAD cDNA (construct **7TP**) or a full-length Eucalyptus CAD cDNA (construct **E**).

Two other constructs were designed associating antisense sequences to tissue-specific promoters: a full-length tobacco CAD cDNA under the control of the GRP1.8 promoter (construct **G**) and a 1 kb 5′ fragment of tobacco CAD cDNA associated with a Eucalyptus CAD promoter (proA CAD, construct **AT**).

Comparative efficiency of the different antisense constructs on CAD activity reduction

Two different populations of control plants were analyzed at the *in vitro* stage: regenerated untransformed plants and plants transformed with a GRP 1.8 promoter associated with a *uid A* gene. The values of CAD activity for the two populations being similar (transformation events have no incidence on CAD activity), they were pooled and treated as one and the same population. The mean value of CAD activity for the control population was 1216 pkatals/mg protein with a standard deviation of 304 pkatals/mg protein. For each individual transgenic line, reduction of CAD activity was expressed as the % of CAD activity compared to the mean value of the control population (represented as 100% by the straight solid line on Fig. 2) and the standart deviation between the 3 replicates was about 1 to 2%.

Effect of antisense homologous constructs driven by constitutive promoters. Using the CaMV 35S promoter driving a partial or a full-length antisense CAD cDNA, transgenic plants inhibited for CAD activity were regenerated (Fig. 2: **T, 3P**). In the first case (construct **T**), 78% of the transformed plants displayed a significant reduction of CAD activity (evidenced by values lower than the control minus standard deviation), while for construct **3P**, 65% of the transformed plantlets were down regulated for CAD activity. The lowest level of residual CAD activity with construct **T** was 14% (transformant **T37**). Construct **3P** allowed a lower level of CAD activity to be reached with transformant 3P27 which displayed 8% residual CAD activity.

The **7TP** population of transgenic tobacco plants carrying the CaMV 35S-DE promoter associated to the full-length CAD cDNA showed the highest general level of reduction of CAD activity: indeed, for all transformants, CAD activity was below 70% residual activity. However, the lowest residual activity (12% transformant 7TP5) was similar to the lowest values obtained with the CaMV 35S promoter.

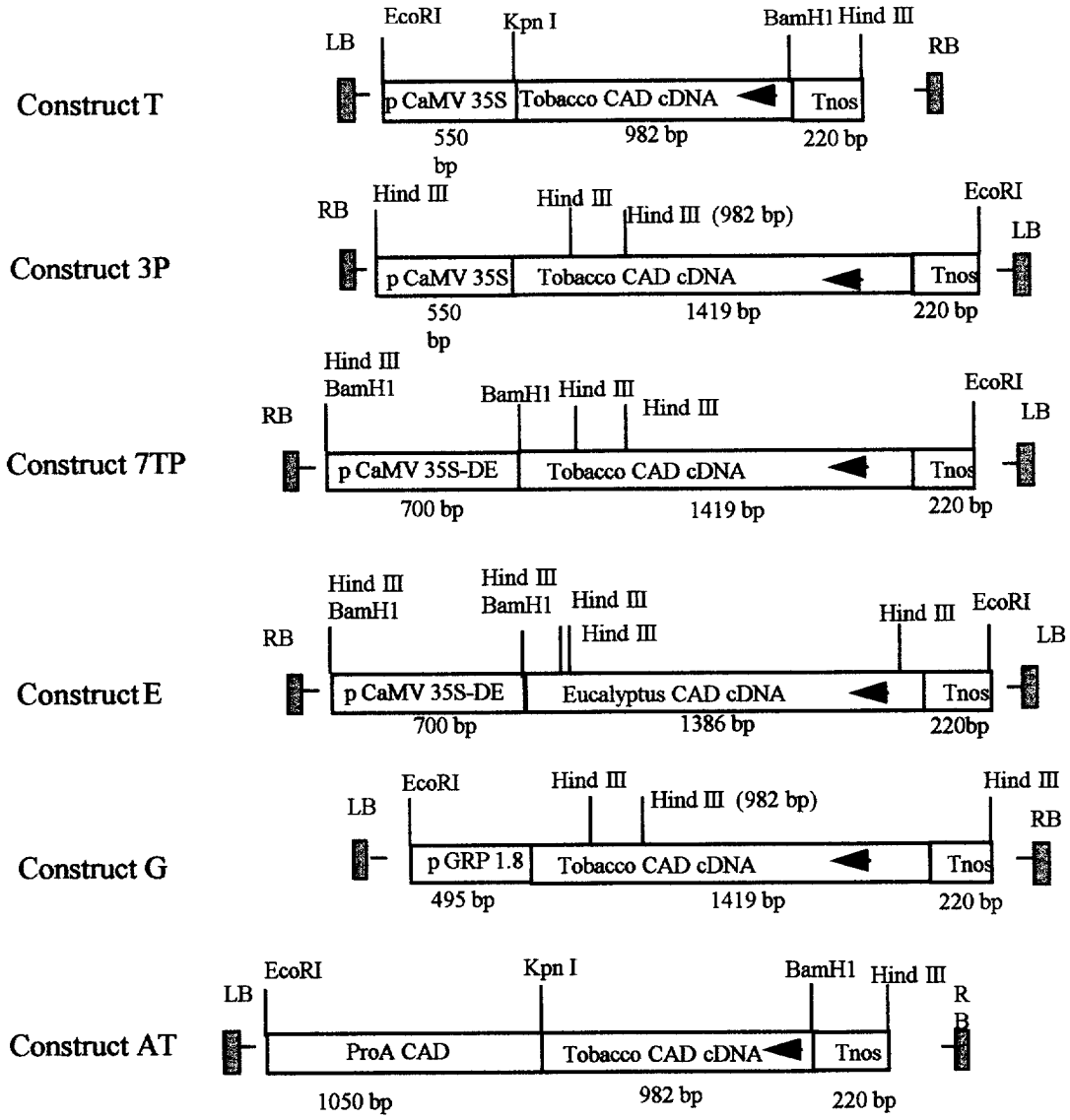

Fig. 1. Schematic physical map of CAD antisense constructs.

Effect of antisense heterologous constructs driven by constitutive promoters. In contrast to 7TP population (CaMV 35S-DE promoter driving a tobacco CAD cDNA in antisense orientation), CAD activity in plants transformed with the antisense Eucalyptus CAD cDNA under the control of the CaMV 35S-DE promoter remained similar to those of the control population (Fig. 2 **E**). Despite a high level of homology (75% nucleic acid identity), the heterologous cDNA was poorly efficient in inhibiting the endogenous tobacco CAD gene expression.

Effects of tissue-specific promoter-driven antisense CAD constructs. The GRP 1.8 promoter, isolated from bean by Keller *et al* [14] and specifically active in protoxylem tracheary elements of vascular tissues was associated to a full-length tobacco CAD cDNA in reverse orientation. The CAD activity of G population was on average quite similar to the control (Fig. 2 **G**). Only transformants G70 and G72 (50 and 72% residual CAD activity respectively) were significantly down regulated.

The Eucalyptus CAD promoter containing the required cis element for tissue-specific expression was already shown to be active in tobacco and poplar, with a preferential expression in lignifying zones [[15], Lauvergeat, pers. com.]. By using a 1 kb sequence of this promoter, the CAD activity of the different transformants was not significantly different from control plants (Fig. 2 **AT**). This CAD promoter sequence as well as the GRP 1.8 promoter did not allow the levels of reduction obtained with the constitutive CaMV 35S promoters to be reached.

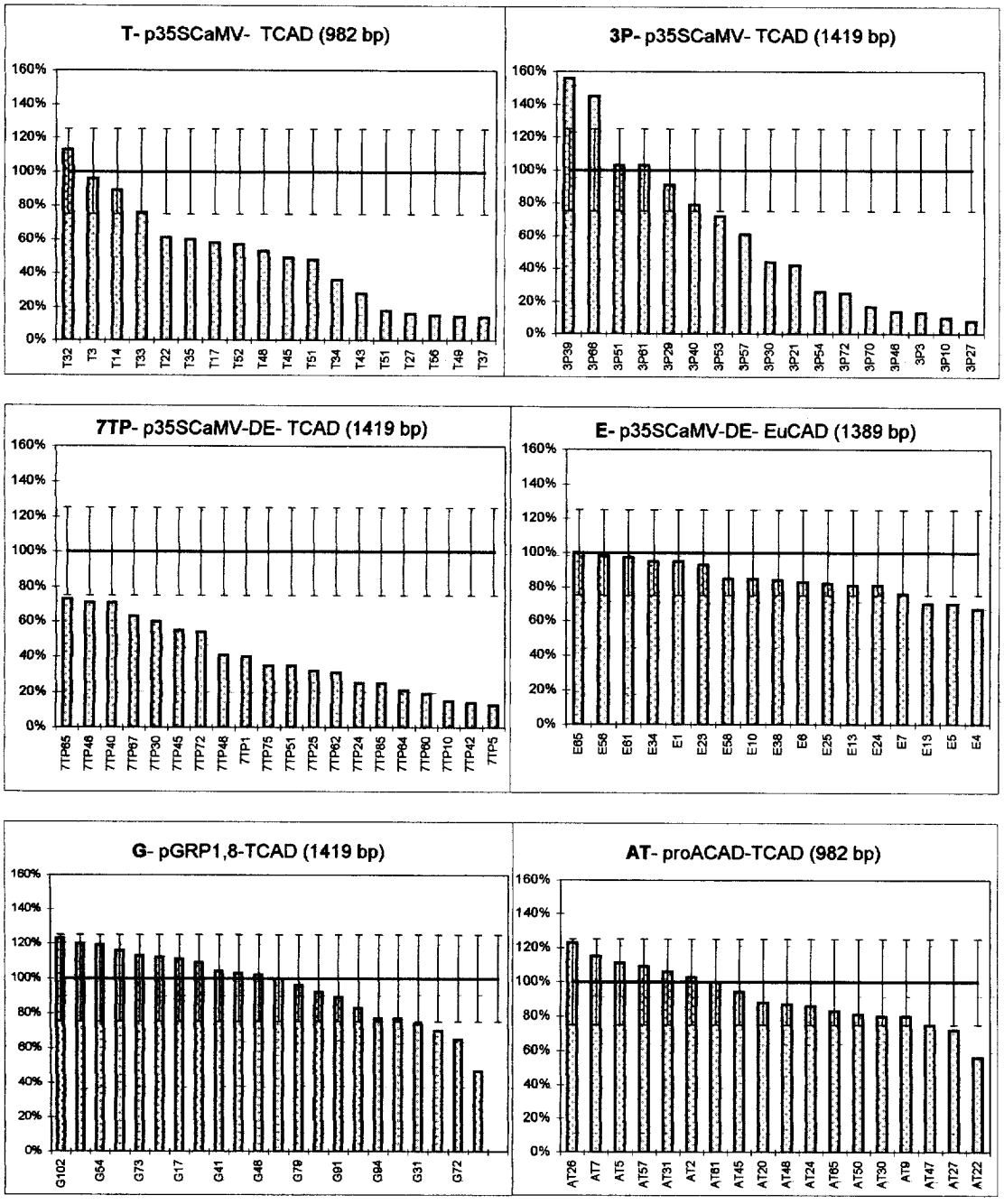

Fig. 2. CAD activity for *in vitro* antisense CAD transformed tobacco plants. Reduction of the CAD activity is expressed as the % activity compared to the mean value of the control population (represented here by the straight line), measurements were carried on three replicates. Plants were transformed with: (T) Partial tobacco CAD cDNA under control of CaMV 35S promoter. (3P) full-length tobacco CAD cDNA under control of CaMV 35S promoter. (7TP) full-length tobacco CAD cDNA under control of CaMV 35S -DE promoter. (E) Eucalyptus CAD cDNA under control of CaMV 35S-DE promoter. (G) full-length tobacco CAD cDNA under control of GRP 1.8 promoter. (AT) partial tobacco CAD cDNA under control of Eucalyptus CAD promoter.

Molecular analysis

For the transformants exhibiting the stronger CAD down regulation, transgene integration loci number was evaluated by kanamycin resistance segregation ratios: 2 loci were evidenced for 3P27, 7TP5, 7TP10 and 3 loci for T37, 3P10, 7TP42. Southern blot experiments were performed after digestion of genomic DNA with EcoRI (one restriction site in the different T-DNAs). In accordance with segregation analysis,

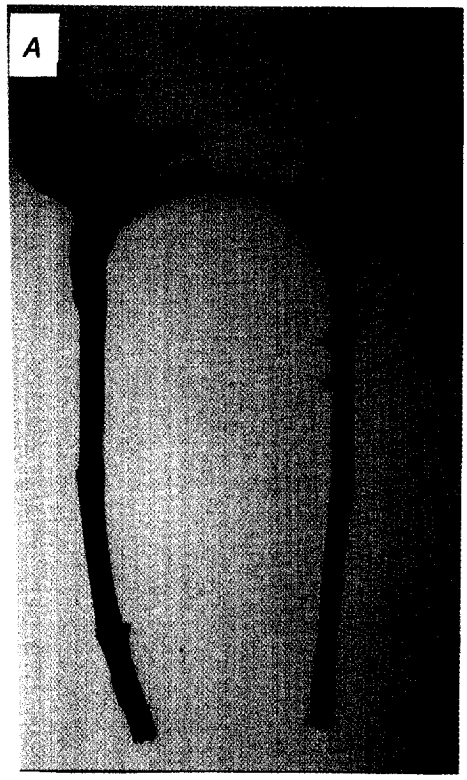

Fig. 4. Red xylem coloration of transgenic tobacco plants with a reduced CAD activity. (A) after two weeks acclimation in the greenhouse. (B) at the adult stage

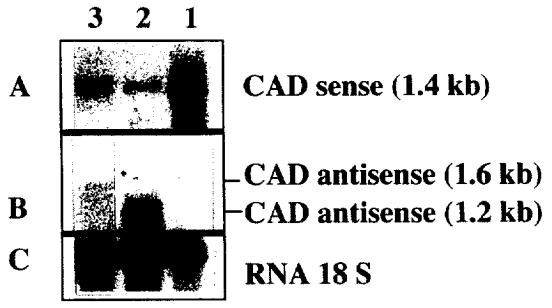

Fig. 3. Northern analysis of selected antisense CAD transformants. Endogenous gene and transgene expression in stems of transgenic and control plants. (A) antisense single strand RNA probe. (B) sense single strand RNA probe. (C) ribosomic RNA 18S probe. 1: Control, 2: T37, 3: 7TP5

hybridization with a CaMV 35S promoter labelled probe revealed (data not shown) the presence of multiple copies of transgenes for all the tested transformants.

In parallel to CAD activity measurements, steady state levels of endogenous and antisense CAD mRNAs were determined using Northern hybridization with riboprobes. Reduced levels of endogenous CAD mRNA as compared to the control were observed for CAD down regulated plants (T37, 7TP5) (Fig. 3). On these two lines, the reduction was well in agreement with the level of CAD activity, particularly for T37 (8% residual CAD activity) which exhibited the weaker endogenous mRNA signal and the highest level for antisense transcript.

Correlation between red xylem coloration and a strong reduction of CAD activity during plant growth

The red xylem coloration is associated with very low residual activity at the *in vitro* stage

One of the main features of transgenic plants with a reduced CAD activity is the red coloration of their xylem [Fig. 4(A)]. This coloration was first observed by Halpin *et al* [7]. for greenhouse grown plants with 7% residual CAD activity. In our case, this coloration was already detectable (without stem dissection) at the *in vitro* stage. Only one of the primary transformants of population T, transformant T37 (14% CAD activity), exhibited a red coloration of the xylem while other transformants (T49, T56) with only subtly higher levels of CAD activity had noncolored xylem.

Within populations 3P and 7TP only the plants exhibiting the lowest levels of CAD activity exhibited this red coloration: 3P3, 3P10, 3P27 and 7TP5, 7TP10, 7TP42 respectively.

None of the transgenic plants carrying constructs under the control of tissue-specific promoters showed a red coloration of the xylem. The detection of this coloration at the *in vitro* stage seems therefore to be a good indicator of strong reductions of CAD activity and could possibly be used as a rapid screening test for transgenic plants with a reduced CAD activity in other species.

Changes in xylem coloration during development as a diagnosis of silencing phenomena. Seven primary transformants (T37, 3P3, 3P10, 3P27, 7TP5, 7TP10, 7TP42) selected on the basis of their low CAD activity, and the red coloration of their xylem, were transferred to the greenhouse and grown until flowering. At the adult stage, the red coloration was either uniform as in the case of transformant T37, or in stripes [Fig. 4(B)], segments and colored patches being present beneath the junction of petiole and stems for the others clones.

Within the same transformant, the red phenotype appears to be developmentally regulated presumably depending on lignin deposition: the xylem in the apical part of the stem is green and the red coloration gradually appears with the formation of the secondary xylem in older tissues. At the adult stage, the red coloration was not only present in stems but also in the stele of primary roots.

Depending on the clones, the red coloration (uniform or with specific profiles) was maintained at the adult stage (T37, 7TP5, 7TP42) or was only present during the first 3 weeks of growth and was lost at the mature stage of the primary transformants (3P3, 3P10, 3P27, 7TP10). This gradual disappearance of the red coloration was associated with an increase in CAD activity (as shown in Fig. 5) for 3P3, 3P10, 3P27 and 7TP10 during growth. As an example, transformant 3P27 which had the lowest CAD activity at the *in vitro* stage (8% residual CAD activity) displayed 56% of CAD activity at the preflowering stage. In contrast, three selected plants still exhibiting the red phenotype maintained a strong reduction of CAD activity at the mature stage: T37 (8%), 7TP5 (11%) and 7TP42 (11%). Progeny was studied for these 3 primary transformants and the same modifications in xylem associated with low level of CAD residual activity (8%) remained stable for F1 generation for T37. Concerning 7TP5 and 7TP42, their typical pattern of red xylem coloration in stripes were not observed in all the progeny.

Among these transformants, transformant 7TP5 had a specific pattern of red xylem coloration which is displayed in regular stripes alternating with well defined uncolored zones. In order to explain this pattern of coloration, CAD activities were compared for the dissected xylem of control plants, the red xylem of T37, the red stripes (Xr) and the uncolored (green) stripes (Xg) of the xylem of 7TP5. Similar results were obtained using sinapyl aldehyde or coniferyl aldehyde as substrates for the measurement of CAD activity. As shown in Fig. 6, levels of CAD activity in the xylem of control plants presented high values (3400 pkatals mg^{-1}). It is in the homogeneously red xylem of the transgenic line T37 that the extent of CAD down regulation is the highest (3 to 4% residual CAD

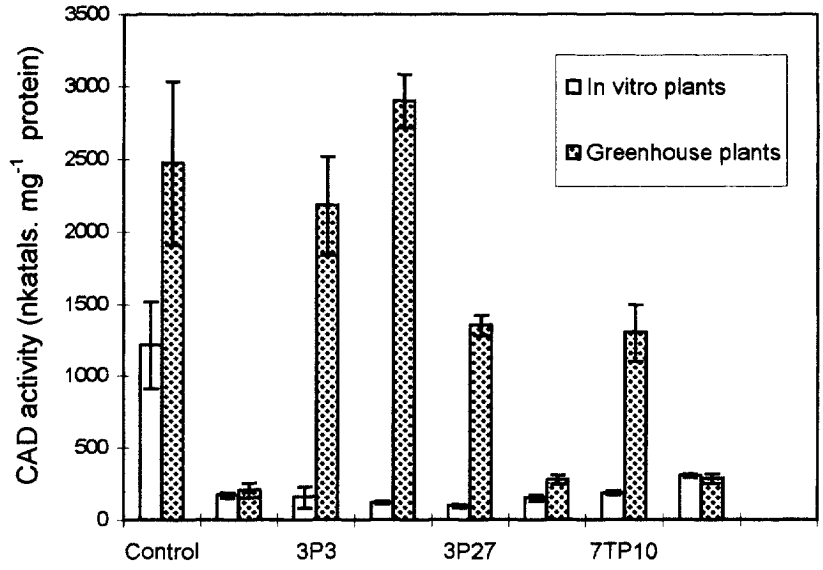

Fig. 5. Comparison of residual CAD activities between 5-week-old *in vitro* plantlets (empty bars) and mature greenhouse grown selected plants (shaded bars). Measurements were carried on at least three plants per line, with sinapyl aldehyde as a substrate.

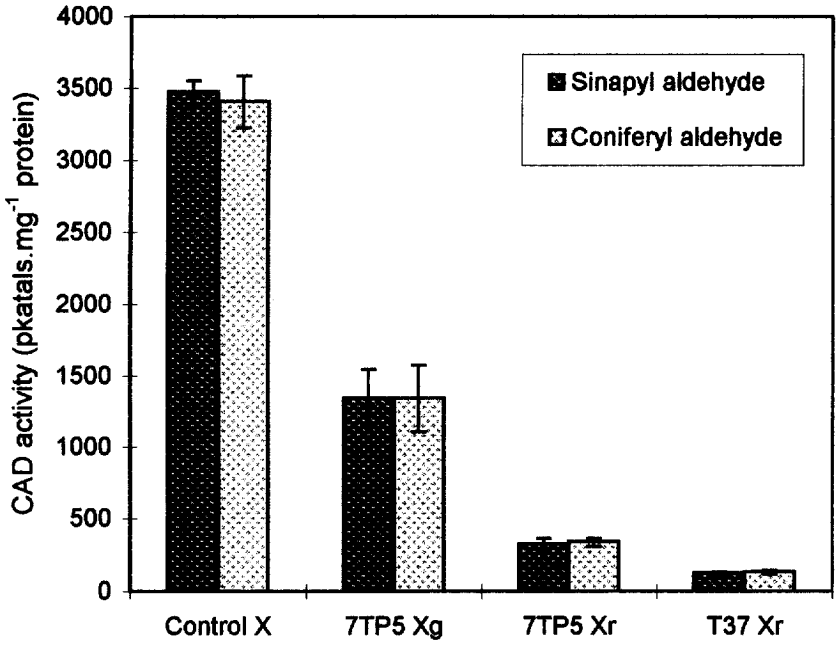

Fig. 6. CAD activity in the xylem of the middle part of stems of transgenic and control tobacco plants. Coniferyl aldehyde and sinapyl aldehyde were used as substrates. At least three plants per line were analysed. X = xylem, Xg = green xylem, Xr = red xylem.

activity). Concerning 7TP5, CAD activity in the green stripes was higher (40% residual CAD activity as compared to the xylem control value) than for the red stripes (9% residual CAD activity). The difference in xylem coloration is therefore correlated to the level of CAD activity.

DISCUSSION

Using various CAD antisense constructs, we showed that a strong inhibition of CAD activity (8 to 11% residual CAD activity) was only achieved in our hands, with homologous antisense CAD cDNAs

driven by constitutive promoters. The CaMV 35S promoter is known to be functional in different plant organs [16] and is nowadays the most widely used promoter for the RNA antisense strategy [reviewed in [17]]. The tandem duplication of enhancer elements localised in the −343 to −90 pb region of this promoter may enhance (up to 10 fold) the expression of genes placed under its control [13]. The proportion of CAD down regulated transformants increased when the CaMV 35S-DE promoter was used. However, the maximum CAD inhibition reached was comparable for both CaMV 35S-DE and CaMV 35S.

Levels of CAD down regulation were quite similar for constructs T and 3P, both under the control of the CaMV 35S promoter but differing by the length of the cDNA sequence. According to the litterature, there is no rule about the relative efficiency of different subgenomic fragments or full-length coding regions for inhibition by an antisense mechanism [17]. For the inhibition of cOMT gene expression [18], the full-length cDNA was the most efficient at the adult stage. In our experiments, the fragment-length of the homologous cDNA was not found to be a crucial factor.

Although the use of tissue-specific promoters in the antisense RNA strategy already proved to be successful [19–21], in our case, the use of tissue-specific promoters did not result in a strong inhibition of CAD activity. In poplar, modifications of lignins were obtained for plants with 30% residual CAD activity [12], however, the results of Halpin *et al* [7] clearly demonstrated that the impact of a reduction of CAD activity on tobacco lignin characteristics was only significant with residual CAD activity below 20%. The levels of reduction obtained in our case with the GRP1.8 and proACAD promoters were not sufficient to induce such effects on lignin and this result was confirmed by the absence of a red coloration of the xylem of these plants. The Eucalyptus proACAD promoter coupled to the GUS gene has already proved to be functional in a heterologous context [15] and the same kind of result has also been demonstrated for other lignification genes [22]. It is however possible that in a heterologous context the tissue-specific promoters are less active due to non-optimal regulatory machinery and future experiments should compare the efficiency of heterologous vs homologous tissue-specific promoters. Indeed, in *Arabidopsis thaliana*, it has been shown that a cinnamate 4-hydrolase homologous promoter is more efficient than the CaMV 35S promoter for driving ferulate hydroxylase antisense constructs (K. Meyer and C. Chapple, pers. com.). Alternatively, the efficiency of the CaMV 35S promoters as compared to the tissue-specific promoters could be not only due to the relative level of transcription but also to the fact that the constitutive expression is earlier and could provide an early pool of antisense mRNAs that are present before initiation of endogenous gene expression which is correlated to tissue differentiation.

When we used heterologous Eucalyptus CAD cDNA, and despite the 75% identity shared with the tobacco cDNA, the corresponding construct was shown to be ineffective for reducing CAD expression in antisense tobacco transformants. A similar result was observed in our laboratory when the Eucalyptus CCR cDNA was introduced in the antisense orientation into tobacco plants (Piquemal *et al.*, unpublished results). Hibino *et al* [11] obtained a 55% reduction of CAD activity in tobacco by using an *Aralia cordata* cDNA (77% homology) and a heterologous cOMT gene also led, in tobacco plants, to a significant but not very high level of inhibition [23]. Moreover, a very strong inhibition with a heterologous cDNA was achieved for the chalcone synthase gene [24] and the granule-bound starch synthase (GBSS) gene [20]. In all these experiments, the homology between the antisense cDNA and the resident gene ranged between 70 and 80% as in our experiments. According to various authors, the efficiency of the antisense strategy is more dependent on the presence of highly homologous short sequences (about 50 bp) than on the overall homology along the whole sequences [16, 25–27]. Two regions of very homologous sequences (53 and 70 bp with respectively 94 and 91% identity) were identified in the 5′ end of tobacco and Eucalyptus CAD cDNAs. The presence of these highly homologous sequences does not appear to be sufficient in our case for a good efficiency of the heterologous construct. Other factors might be involved such as the position of the homologous regions and/or their relative size as compared to the full-length cDNA.

The lowest level of residual CAD activity measured in the stems of the tested transgenic plants ranged between 8 and 11%. This level is similar to that already obtained by Halpin *et al* [7] in tobacco (7% residual CAD activity). It is interesting to note that up to now and in contrast to the down regulation of other lignification genes such as cOMT [18] it has been impossible to reduce the residual CAD activity through genetic manipulation below 7–10% in stems [7, 11, 12]. However, in gymnosperms, a pine «cad null» mutant has been recently characterized with 1% CAD activity [28]. The existence of such a mutant demonstrates that it is theoretically possible to recover viable plants with levels of CAD activity lower than 7%. The existence of various types of aromatic alcohol dehydrogenases with wide substrate specificities (exhibiting poor or no sequence homology with CAD) was already evidenced in angiosperms [29]. The basal level of CAD activity observed in our experiments and others could be attributed to some of these alcohol dehydrogenases able to use sinapyl aldehyde and/or coniferyl aldehyde as substrates.

Only three of our selected transgenic plants (T37, 7TP5, 7TP42, 8 to 11% residual CAD activity) maintained a low level of CAD activity during growth and maturation. Results of lignin analysis for these transformants indicated that the most significant changes in lignin composition (e.g. integration of cin-

namaldehydes in lignins, increased lignin extractability by mild alkali treatment) were obtained for line T37 which displays 8% residual CAD activity and is characterized by a uniform red coloration that was persistant until maturation [10].

In our case, all the primary transformants with a significant down regulation of CAD activity exhibit multiple copies of the transgene, but in a previous study, with construct T, Halpin *et al.* [7], obtained a similar inhibition level (8% residual CAD activity) for a transgenic line containing a single copy of the transgene. In addition, 7TP5 and 7TP42, containing respectively 2 or 3 integrations of the same antisense construct, exhibited strictly the same CAD down regulation. Thus, it is difficult to establish a correlation between the transgene copy number and the extent of down regulation. We hypothesize that the position effect may play a more important role than the copy number.

A number of the *in vitro* selected primary transformants showed a decreased level of reduction of CAD activity during growth in the greenhouse. For some of the transformants the down regulation of CAD activity is not uniform in the different parts of the stem. Our data illustrate silencing phenomena occurring during development and/or specifically affecting various sectors of one and the same organ. They also confirm the unpredictability of the extent of these silencing effects since, in our hands, multiple copy transformants exhibited a good stability of CAD down regulation and others with the same number of transgene copies exhibit a significant relative increase in CAD activity during plant development. Position effects are likely important in these specific and divergent responses. Atanassova *et al* [13] and Piquemal *et al* [30] also observed a relaxation of the antisense effect on cOMT and CCR activities (respectively) during the transfer of the transgenic plants from *in vitro* culture conditions to the greenhouse and during the development of the plants. These observations are likely due to transgene silencing phenomena. Several theories have been proposed to explain the mechanisms underlying gene or transgene silencing. The gene inactivation process could be due (i) to transcriptional silencing induced by DNA-DNA interactions [31, 32]; (ii) to post-transcriptional silencing based either on DNA-DNA pairing between homologous loci leading to the synthesis of aberrant RNAs and to subsequent degradation of homologous RNAs [33, 34], or to specific mechanisms of transcript degradation that would be activated when gene products (transgenic and/or endogenous) accumulate above a certain threshold level [35]. In addition, Elmayan and Vaucheret [36] have recently reported that post-transcriptional silencing affected hemizygous and haploid transgenic tobacco plants carrying a single copy of a *uidA* gene under the control of a CaMV 35S promoter with a duplicated enhancer. In that particular case, the DNA-DNA pairing cannot occur and the process of inactivation is triggered by elevated levels of *uid A* transcripts due to the use of a strong promoter.

In conclusion, the most efficient system for good inhibition of CAD activity was homologous cDNA associated with a constitutive promoter and integrated in multiple copies, however, paradoxically, a strong promoter that is too strong can compromise the stability of the antisense effect during plant growth. In view of a future extension of the CAD antisense strategy to economically important crops and woody species, we would recommend at the moment the use of homologous cDNA and constitutive promoters. However, it will be undoubtedly interesting to further probe the potential use of tissue-specific promoters to restrict the expression of the transgenes to selected tissues through the use of promoters of genes that are expressed earlier in the lignin biosynthetic pathway like PAL (phenylalanine ammonia lyase), C4H (cinnamate-4-hydroxylase) or 4CL (4-coumarate ligase) promoter(s). Alternatively, the determination of specific sequences (cis elements) controlling gene expression in the xylem could improve the antisense strategy for lignin modulation using xylem-specific sequences associated with enhancer elements of a constitutive promoter. In this way, the detailed characterization of CAD and CCR promoters is currently undergoing in our laboratory.

EXPERIMENTAL

Construction of chimeric genes

A full-length (1.4 kb) and a 1kb EcoRI-Hind III 5′ fragment of tobacco CAD cDNA isolated from plasmid pTCAD 19 [6] were provided by C. Halpin (Zeneca Seeds) in PJR1, a pUC18-derived vector containing the CaMV 35S promoter (528 bp) and the 3′ terminator of the nopaline synthase gene [37, 7]. For both constructs, the expression cassettes were inserted in binary vector pBin 19 [38] as EcoRI-Hind III fragments.

For the antisense construct driven by tissue-specific promoter GRP 1.8 (Glycine Rich Protein), full-length tobacco CAD cDNA was isolated from plasmid pTCAD 19 as an EcoRI fragment, blunt ended with the Klenow fragment of DNA polymerase and cloned into the Sma/SstI sites of a pBin19 derived vector containing the GRP.1.8 promoter and the 3′ terminator of the nopaline synthase (kindly provided by B. Keller, Switzerland). The final plasmid was named pGRPCAD.

A 1 kb fragment of Eucalyptus CAD promoter (proACAD) [15] was amplified by PCR using primers including either an EcoRI or a Kpn I restriction site. Following EcoRI-KpnI digestion, the promoter fragment was inserted into a modified pBin 19 derived vector (containing the CaMV 35S promoter (528 bp), a pUC 18 polylinker sequence and the 3′ terminator of the nopaline synthase (J. Ray, Zeneca Seeds)) in place of the CaMV 35S promoter and the resulting

vector was named proAJRI. Tobacco CAD cDNA in antisense orientation was then inserted into proAJR1 as a KpnI-BamHI fragment.

Full-length tobacco and Eucalyptus CAD cDNAs in antisense orientation and associated with the 3′ terminator of the nopaline synthase were obtained as BamHI-EcoRI fragments from pGRP CAD and inserted into pLBR19 (provided by L. Jouanin, INRA, Versailles), a pCa2 derived vector (described by Kay *et al.*, [17]) containing the CaMV 35S promoter with a duplicated enhancer, a polylinker sequence and the CaMV 35S terminator. For both constructs, the promoter (p CaMV 35S-DE), the cDNA in antisense orientation and the 3′ terminator of the nopaline synthase gene were ligated in Kpn-EcoRI digested binary vector pBin 19. All constructs were transferred to tobacco through *Agrobacterium tumefaciens* mediated transformation of leaf discs.

Plant transformation and characterization of transformants

Leaf discs of tobacco (*Nicotiana tabacum* L. cv. Samsun) were transformed via *Agrobacterium tumefaciens* according to the procedure of Horsch *et al.*[39]. For each construct, at least 17 independent kanamycin-resistant shoots were regenerated. Transformants regenerated on kanamycin medium were screened for CAD activity. The presence of the transgenes was confirmed by PCR using primers corresponding to the different promoters and the 3′ nopaline synthase terminator and the transformed *in vitro* plantlets were screened for CAD activity. Once tested for CAD activity, plants of interest were transferred to the greenhouse and allowed to self pollinate. F1 seeds were harvested and tested for segregation of kanamycin resistance on a germination medium containing 500 mg/l kanamycin. After 4 weeks of culture, the number of F1 sensitive or resistant plantlets was scored and their ratio compared to theoretical segregation ratios using a χ^2 statistical test. Transgene copy number was confirmed by Southern blotting. Northern analysis and additional CAD assays were performed on greenhouse grown plants at the emergence of the flower buds.

CAD assays

CAD assays were performed on 2 cm basal stem sections of 5-week-old *in vitro* transformed and control plants or from 1 cm sections in the mid part of the stem of greenhouse adult plants. Proteins were extracted as described by Goffner *et al.* [40]. CAD activity was assayed following the reduction of sinapyl aldehyde and, in some experiments, of coniferyl aldehyde. The reaction was monitored by the change of absorbance at 340 nm due to the disappearance of NADPH and of the cinnamaldehyde. The assay was carried out in 0.5 ml reaction mixture containing 100 mM KH_2PO_4/Na_2HPO_4 (pH 6.25), 50 μM sinapyl aldehyde ($E = 19{,}1.10^3\,M^{-1}\,cm^{-1}$) or 50 μM coniferyl aldehyde ($E = 18.5.10^3 M^{-1}\,cm^{-1}$), 100 μM NADPH and 50 μl of protein sample. Protein concentration was determined by the method of Bradford [41] using the Bio-Rad dye binding reagent. The mean CAD value of three individual plants from each primary transformant was compared to the mean value of the control population.

For CAD analysis on different tissues, 5 cm long stem section (middle part of the stem) were dissected, the epidermis and cortex tissue were peeled off. Xylem strips were then carefuly removed from the pith and frozen in liquid nitrogen.

Southern and northern hybridizations

Genomic DNA was prepared from frozen leaf tissues using a CTAB (cetyltrimethyl ammonium bromide) procedure [42]. Ten μg DNA were digested with EcoRI (one restriction site within the T-DNA) in the presence of 20 mM spermidine. Gels were blotted into Nytran N membrane (Schleicher and Schull) according to the manufacturers instructions. A α^{32}P-dCTP labeled CaMV 35S promoter sequence (550 bp) was used as a probe. Hybridization was performed overnight at 65°C in 3 × SSc, 0.5% SDS, and 25% low fat milk. Membranes were washed at 65°C for 15 min in 1.5 × SSc, O.5% SDS, then for 20 min in 1.5 × SSc, O.5% SDS and autoradiographed.

Total RNA was prepared from stems of greenhouse grown mature plants as described by Jones *et al.* [43]. Total RNA (25 μg) was run on 1% agarose gel using the glyoxal/DMSO method [44] and transferred to Hybond N+ membrane (Amersham). Filters were hybridized with ^{32}P-labeled CAD single stranded riboprobes using either T3 or T7 polymerase (Promega) to obtain sense and antisense CAD RNA. Prehybridization and hybridization were performed as described in Sambrook *et al.* [44].

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