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Nuclear factors GT-1 and 3AF1 interact with multiple sequences within the promoter of the *Tdc* gene from Madagascar periwinkle: GT-1 is involved in UV light-induced expression

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Abstract Plant secondary metabolites of the terpenoid indole alkaloid (TIA) class comprise several compounds with pharmaceutical applications. A key step in the TIA biosynthetic pathway is catalysed by the enzyme tryptophan decarboxylase (TDC), which channels the primary metabolite tryptophan into TIA metabolism. In *Catharanthus roseus* (Madagascar periwinkle), the *Tdc* gene is expressed throughout plant development. Moreover, *Tdc* gene expression is induced by external stress signals, such as fungal elicitor and UV light. In a previous study of *Tdc* promoter architecture in transgenic tobacco it was shown that the −538 to −112 region is a quantitative determinant for the expression level in different plant organs. Within this sequence one particular region (−160 to −99) was identified as the major contributor to basal expression and another region (−99 to −37) was shown to be required for induction by fungal elicitor. Here, the in vitro binding of nuclear factors to the −572 to −37 region is described. In extracts from tobacco and *C. roseus*, two binding activities were detected that could be identified as the previously described nuclear factors GT-1 and 3AF1, based on their mobility and binding characteristics. Both factors appeared to interact with multiple regions in the *Tdc* promoter. Mutagenesis of GT-1 binding sites in the *Tdc* promoter did not affect the basal or elicitor-induced expression levels. However, induction of the *Tdc* promoter constructs by UV light was significantly lower, thereby demonstrating a functional role for GT-1 in the induction of *Tdc* expression by UV light.

Key words *Catharanthus roseus* · Terpenoid indole alkaloids · *Tdc* · Nuclear factors · UV light

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Introduction

A limited group of plant species produce terpenoid indole alkaloids (TIAs) as part of their secondary metabolism. It is thought that TIAs serve protective functions, like other classes of secondary metabolites such as other types of alkaloids (Hartmann 1991) or phenylpropanoid-derived phytoalexins (Dixon and Paiva 1995). Indeed, some experimental evidence suggests that TIAs are involved in defence by providing protection against microbial infection, herbivory or UV radiation (Aerts et al. 1991; Luijendijk 1995; Thomas et al. 1995). However, their exact role in the physiology of the plant remains to be elucidated. Research interest in TIA metabolism is mainly motivated by the pharmaceutical applications of the TIA alkaloids. The tropical plant *Catharanthus roseus* (Madagascar periwinkle, family Apocynaceae) produces, among others, vincristine and vinblastine, which are used in anti-tumour therapies, and ajmalicine and serpentine, which are used in the treatment of heart and circulatory diseases (Kutchan 1995).

The enzyme tryptophan decarboxylase (TDC; EC 4.1.1.28) operates in TIA biosynthesis by decarboxylating the amino acid tryptophan to form tryptamine. Tryptamine is then condensed with secologanin by the enzyme strictosidine synthase (STR) to form strictosidine, which serves as the universal precursor for all other TIAs found in plants (reviewed by Meijer et al. 1993a; Hashimoto and Yamada 1994). Biosynthesis of the terpenoid secologanin starts with the conversion of geraniol into 10-hydroxy-geraniol, which is catalysed by geraniol-10-hydroxylase (G10H), a cytochrome P-450 mono-oxygenase whose function is dependent on NADPH:cytochrome P-450 reductase (CPR) (reviewed by Meijer et al. 1993a).

At present, cDNA and genomic clones for the TIA biosynthetic genes *Tdc* (De Luca et al. 1989; Goddijn

et al. 1994), *Str* (McKnight et al. 1990; Pasquali et al. 1992, 1999) and *Cpr* (Meijer et al. 1993b; Lopes-Cardoso et al. 1997) have been isolated from *C. roseus*. In cell suspensions, the *Tdc*, *Str* and *Cpr* genes are coordinately induced by fungal elicitors (Pasquali et al. 1992; Roewer et al. 1992; Meijer et al. 1993b), and repressed by auxin (Goddijn et al. 1992; Pasquali et al. 1992; Meijer 1993). Furthermore, the expression of *Tdc* (Goddijn et al. 1995), *Str* (Pasquali et al. 1992; Ouwerkerk and Memelink 1999b) and *Cpr* (Meijer et al. 1993b) is higher in roots of *C. roseus* plants than in leaves and stems, which is consistent with the relative TIA levels in these organs (Hoekstra 1993). Taken together, these results suggest that genes involved in TIA biosynthesis are coordinately regulated by the same signal transduction pathway. Constitutive expression of the *Tdc* coding region under control of the strong cauliflower mosaic virus (CaMV) 35S promoter enhanced TDC enzyme activity up to tenfold in transgenic *C. roseus* callus (Goddijn et al. 1995), indicating that TDC enzyme levels are determined mainly by the expression level of the *Tdc* gene. *Tdc* promoter sequences were shown to confer reporter gene activity in leaves, stems and roots of transgenic tobacco plants (Goddijn et al. 1994). Progressive 5′ deletions gradually decreased promoter strength. A −538 promoter still showed considerable activity. Deletion to −278 had little effect, whereas deletion to −112 reduced promoter activity to low levels. Other loss- and gain-of-function studies with the *Tdc* promoter in tobacco showed that the major contributing enhancer for basal expression lies in the −160 to −99 region (Ouwerkerk and Memelink 1999a). Another region (−99 to −37) was shown to be involved in induction by elicitor. These studies have demonstrated that transcriptional control is an important factor in the determination of TDC enzyme levels in plant tissues, and that multiple promoter regions determine the expression level. The *Tdc* promoter constructs are regulated by fungal elicitor (Ouwerkerk and Memelink 1999a) and UV light (our unpublished results), as is also true for the endogenous *Tdc* gene in *C. roseus* (Pasquali et al. 1992). Similarly, reporter gene expression driven by the *Str* (Pasquali et al. 1999) and *Cpr* (Lopes-Cardoso et al. 1997) promoters responds to elicitor in transgenic tobacco, in agreement with the responsiveness to elicitor of the corresponding genes in *C. roseus*. Although tobacco and *C. roseus* are not closely related, apparently some mechanisms that regulate the TIA biosynthetic genes *Tdc*, *Str* and *Cpr* are conserved between the two species. Therefore we postulate that these genes are regulated by a defence signal transduction pathway that is conserved among plant species.

The aim of this study was to obtain insight into the DNA binding activities that interact in vitro with the *Tdc* promoter sequence from position −572 to −37. We therefore carried out electrophoretic mobility shift assays using nuclear extracts from *C. roseus* and tobacco. Our results show that the previously described factors GT-1 (Green et al. 1987) and 3AF1 (Lam et al. 1990) bind to several *Tdc* promoter fragments. Interestingly, GT-1 was also shown to interact with important enhancer regions in the *Str* (Pasquali et al. 1999) and *Cpr* (Lopes-Cardoso et al. 1997) genes. To investigate the role of GT-1 in regulating the activity of the *Tdc* promoter in vivo, we employed a strategy in which multiple binding sites in an important enhancer were mutagenized. Analyses of mutant promoter constructs in transgenic plants showed no effect on basal or elicitor-mediated expression of the *Tdc* promoter. However, induction of the *Tdc* promoter by UV light was decreased, demonstrating that GT-1 functions in the regulation of the *Tdc* gene by UV light.

Materials and methods

Electrophoretic mobility shift assays (EMSA) and methylation interference analysis

Nuclear extracts were prepared from mature tobacco leaves and from 1-week-old, suspension-cultured *C. roseus* cells as described by Green et al. (1991). All EMSA reactions contained 3 μg of poly-(dIdC)-poly-(dIdC) (Pharmacia LKB Biotechnology) and 0.1 ng of ^{32}P end-labelled probe (10^8 cpm/μg) in nuclear extraction buffer (Green et al. 1991). The amounts of competitor DNAs and protein extracts used are indicated in the text or figures. A map of the *Tdc* promoter with relevant restriction sites is presented in Fig. 4. Coordinates are given relative to the transcription start site. Fragments used for competition experiments or as probes were recovered from pBluescript II SK$^+$ (Stratagene) or other suitable cloning vectors by cleavage at flanking restriction sites and isolation from polyacrylamide gels. The binding reactions were incubated for 30 min at room temperature. For supershift assays, 2 μl of rabbit antiserum was added after 20 min, and incubation was continued for an additional 10 min. Rabbit polyclonal anti-GT-1b antiserum was raised against the His-tagged protein, isolated using Ni^{2+} affinity chromatography from *Escherichia coli* expressing GT-1b from the corresponding cDNA clone (Pasquali et al. 1999). DNA-binding reactions were loaded onto 5% acrylamide/bisacrylamide (37.5:1) gels with the voltage switched on (10 V/cm); 0.5 × TBE was used as running buffer. The gels were dried on DE81 paper (Whatman) and autoradiographed. Methylation interference assays were performed as described previously (Green et al. 1991; Pasquali et al. 1999).

Vector construction and generation of transgenic plants

All constructs and promoter fragments used are presented in Fig. 9B. The cloning of constructs Δ278, Δ160, Δ99, Δ37 and 4TD-37 was described previously (Goddijn et al. 1994; Ouwerkerk and Memelink 1999a). In construct Δ278m the Gs at positions −212, −209, −208, −158 and −139 in the *Tdc* promoter sequence from −278 to +78 bp were replaced by Ts by using the primers 5′-CGATTAAAAAAAAAAAAAATTAAAAAC-3′ and 5′-GATATTTTATATTTAGAAC-3′ in polymerase chain reactions. Construct Δ160m was prepared from construct Δ278m in the same way as construct Δ160 was prepared from construct Δ278 (Ouwerkerk and Memelink 1999a). Tetramerization of TDm and subsequent cloning upstream of the −37 *Tdc* promoter was performed as described previously for construct 4TD-37 (Ouwerkerk and Memelink 1997, 1999a). All the promoter constructs were translationally coupled to the *gusA* reporter gene in the binary vector pBI101.1 as described previously (Goddijn et al. 1994).

The *gusA* constructs were transferred via *Agrobacterium tumefaciens* LBA4404 into tobacco (*N. tabacum* cv. Petit Havana SR1), using the leaf disc transformation procedure (Horsch et al. 1991). Transgenic plants were selected with 100 μg/ml of each of the antibiotics kanamycin, vancomycin and cefotaxime, on Murashige-Skoog medium (Murashige and Skoog 1962) supplemented with

3% sucrose and 0.7% Plant Tissue Culture agar. Growth conditions were 21 °C with a photoperiod of 16 h light (2000 lx) per day.

Treatment of transgenic tobacco plants with UV light, preparation of leaf extracts and *β*-glucuronidase assay

Leaf discs (5 mm diameter) were prepared from 6- to 8-week-old transgenic tobacco plants, grown in tissue culture jars. The UV treatment was performed by placing the leaf discs on wet filter paper in a petri dish and irradiating them for 2 min on a UV illuminator (λmax 312 nm; 28 J per m^2/s). Next the leaf discs were incubated for 24 h in water. Control leaf discs were treated with water under the same conditions. Protein extracts were prepared by homogenization of the leaf discs with a Potter S homogenizer (Braun) in GUS extraction buffer. GUS enzyme activities were measured according to Jefferson (1987) using a Perkin-Elmer LS50B fluorimeter; protein concentrations were determined using the Bradford (1976) protein assay.

Results

The *Tdc* promoter binds GT-1-like nuclear factors from tobacco and *C. roseus*

Previous analysis of *Tdc* promoter derivatives fused to *gusA* in transgenic tobacco showed that deletion of the −278 to −112 region reduces promoter activity to low levels (Goddijn et al. 1994). In addition, a combination of loss- and gain-of-function analyses demonstrated that the TD region (from −160 to −99) contains an enhancer which is the major contributor to the expression level conferred by the *Tdc* promoter (Ouwerkerk and Memelink 1999a). EMSA of fragment HD (−238 to −99) or TD (−160 to −99) with tobacco leaf nuclear extract revealed two complexes that differed in mobility and relative abundance (Fig. 1A). A similar result was

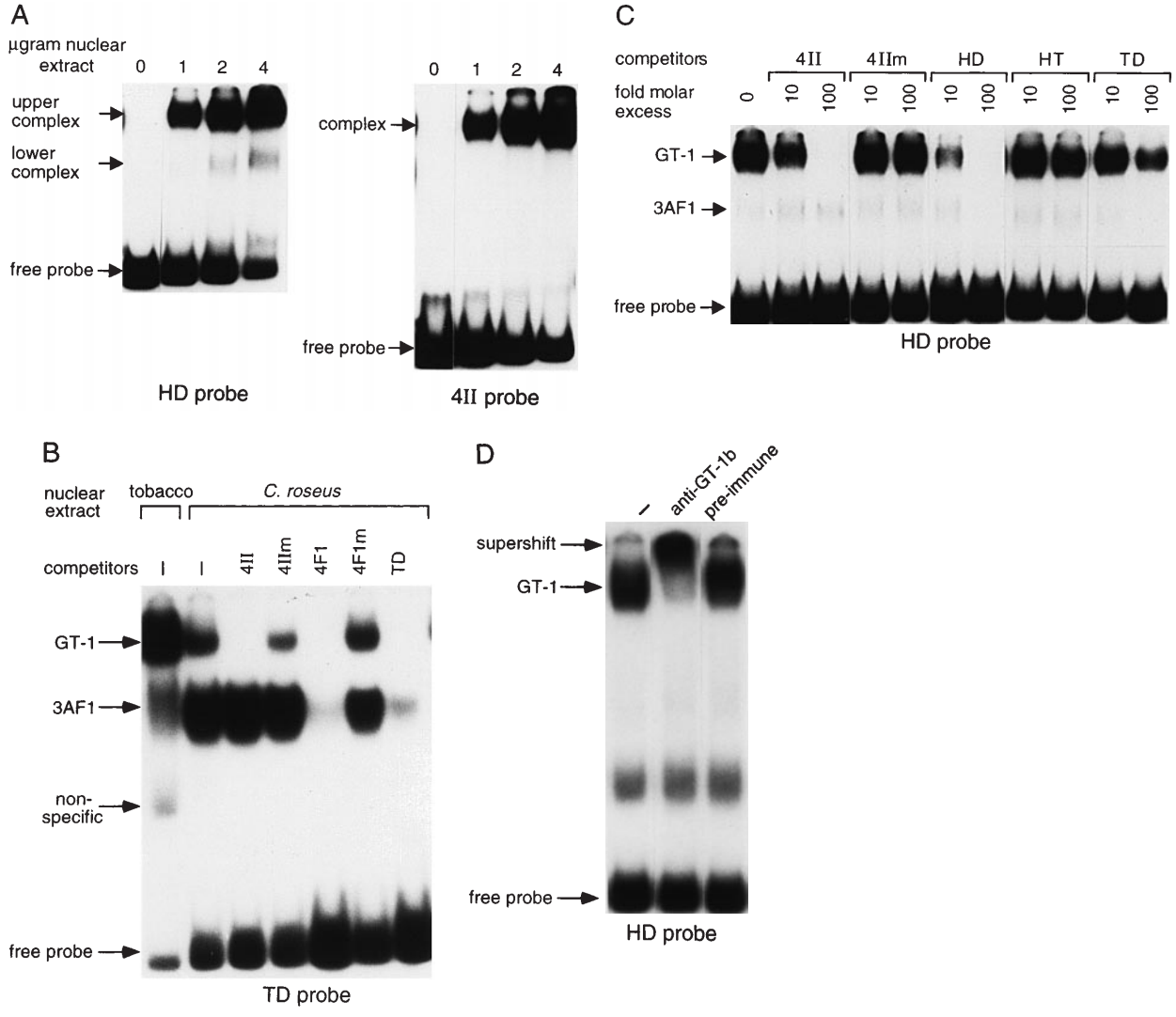

Fig. 1A–D The *Tdc* promoter binds GT-1-like nuclear factors from tobacco and *C. roseus*. **A** Binding of tobacco nuclear proteins to probes HD and 4II. Protein extract was added in the indicated amounts. **B** Probe TD was incubated with 10 µg of *C. roseus* and 4 µg of tobacco nuclear protein. Competitors were added in 200-fold molar excess. **C** Probe HD was incubated with 0.5 µg of tobacco nuclear protein. Competitors were added in the indicated molar excess. **D** Addition of antiserum raised against tobacco GT-1b to a binding reaction containing the probe HD and tobacco nuclear protein causes a supershift. Pre-immune serum was used as a control

obtained using *C. roseus* nuclear proteins extracted from suspension-cultured cells (Fig. 1B).

The low mobility of the upper complex closely resembled that of the GT-1 complex formed with the light-regulatory box II from the promoter of the pea *RbcS-3A* gene, encoding ribulose 1,5-bisphosphate carboxylase (Green et al. 1987). We tested our nuclear extracts for the presence of GT-1 using a tetramer (4II) of the box II sequence (5′-TGTGTGGTTAATATG-3′) as a probe. The complex formed with box II showed the same mobility and abundance as the upper complex which was obtained with *Tdc* probe HD (Fig. 1A; data not shown for the *C. roseus* extract). To confirm that the upper complex represented binding of nuclear GT-1, competition experiments were carried out. As shown in Fig. 1C, the upper complex could be competed out with unlabelled box II tetramer or fragment HD or TD. Similar results were obtained when these competitors were used in EMSAs with 4II as probe (Fig. 2B; and data not shown for *C. roseus* extract). A mutant version (4IIm) of the box II tetramer, containing four copies of the sequence 5′-TGTGTCCTTAATATG-3′, was tested as a control for binding specificity in the competition experiments. The alteration of GG into CC eliminates binding of GT-1 to box II (Green et al. 1988). The 4IIm mutant did not compete for either the upper HD complex or for the GT-1 complex obtained with probe 4II (Fig. 1C, 2B).

To obtain additional proof that GT-1 is binding to the HD fragment, the affinity of the binding activity for antibodies specific for the tobacco GT-1b protein was tested in a supershift assay. Like the GT-1a protein (Gilmartin et al. 1992), GT-1b is a member of the tobacco GT-1 family. It shows 98% sequence identity to GT-1a and the same DNA binding specificity for box II variants (R. J. De Kam, J. Memelink, unpublished results). As shown in Fig. 1D, addition of anti-GT-1b antibodies to the EMSA reaction with the HD probe caused the complex to show a lower mobility, whereas pre-immune serum had no effect. The amounts of antibodies needed to supershift the GT-1 complex formed with HD or box II were identical (results not shown). No supershift was observed (results not shown) with an unrelated protein-DNA complex formed between nuclear ASF-1 and the *as-1* binding site from the CaMV 35S promoter (Lam et al. 1989), demonstrating the specificity of the antibodies.

Based on complex mobility, binding specificity and reaction with anti-GT-1b antibodies, it can be concluded that the nuclear binding activity GT-1 from tobacco interacts with the *Tdc* promoter fragment HD. Incubation with *C. roseus* extracts yielded a complex with identical mobility and specificity.

The *Tdc* promoter contains multiple binding sites for the nuclear factor GT-1

Further characterization of GT-1 binding to the *Tdc* promoter involved testing the HD subfragments HT (−238 to −160) and TD (−160 to −99) as competitors in EMSAs using HD or 4II as probes. As shown in Fig. 1C and 2B for tobacco nuclear extract, subfragments HT and TD competed ineffectively compared with HD. A ten-fold molar excess of HD competed significantly for GT-1 binding, whereas the same amounts of HT or TD gave little competition. Effective competition with TD required a 200-fold molar excess, but this was still insufficient for competition by HT (results not shown). These results clearly demonstrate that the affinity of GT-1 for HD is considerably higher than that for its subfragments HT and TD. Experiments using TD and HT as probes led to the same conclusion (Fig. 2A, C). The upper complexes formed with these probes were competed by 4II and not by the mutant sequence 4IIm, which demonstrates that the complexes result from binding of GT-1. A ten-fold molar excess of 4II or HD competitor was sufficient for nearly complete competition of the GT-1 complex, whereas complete competition with TD required a 100-fold molar excess. The same amount of HT did not fully prevent formation of the labelled complex, indicating that GT-1 exhibits a lower affinity for this fragment. The relative affinities of GT-1 for HD, HT and TD, as determined by competition assays reflect the relative amounts of nuclear extract which are necessary to form similar amounts of GT-1 complexes in EMSAs with these fragments as probes. With probes 4II and HD, a GT-1 complex was visualized with only 0.5 μg of protein extract, whereas probes TD and HT required 4 and 10 μg, respectively (Figs. 1A, 2A, C).

Four other *Tdc* promoter fragments were tested for GT-1 binding in competition EMSA studies with TD and 4II probes. As shown in Fig. 2A and B for tobacco nuclear extract, fragments HS (−572 to −442) and RH (−352 and −238) competed poorly for binding of GT-1. Fragment SR (−442 to −352), however, competed to the same degree as fragment TD, indicating that GT-1 has the same affinity for these latter fragments. Fragment DB (−99 to −44) was the only *Tdc* promoter fragment in this experiment which did not show detectable competition for GT-1 (Fig. 2A, B). Among all *Tdc* promoter fragments tested, HD exhibited the highest affinity for GT-1. Competition experiments using *C. roseus* nuclear extract gave results that were indistinguishable from those with tobacco extract (data not shown).

Methylation interference and DNase I footprinting assays of the pea *RbcS-3A* promoter resulted in the identification of several GT-1 binding sites (Green et al. 1988), from which a consensus binding site was derived (Table 1). The *Tdc* promoter sequence from −575 to +198, which is presented in Fig. 5, was scanned for homology to this consensus sequence. As shown in Table 1 and indicated in Fig. 5, fragments HS, SR, HT and TD contain, respectively, one, three, one and three sequences that show homology to the consensus GT-1 binding sequence GPu(T/A)AA(T/A) (Green et al. 1987).

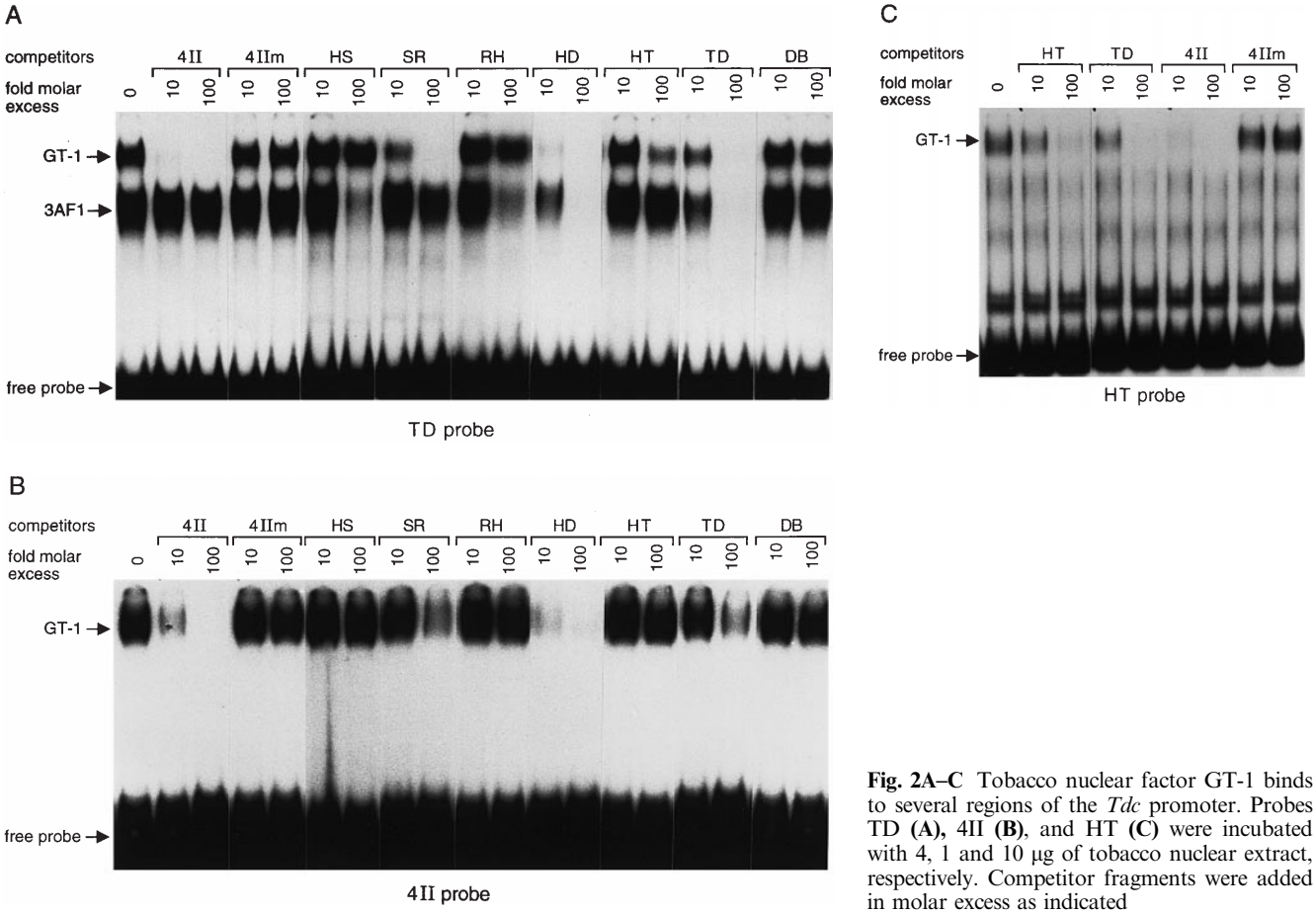

Fig. 2A–C Tobacco nuclear factor GT-1 binds to several regions of the *Tdc* promoter. Probes TD **(A),** 4II **(B)**, and HT **(C)** were incubated with 4, 1 and 10 μg of tobacco nuclear extract, respectively. Competitor fragments were added in molar excess as indicated

The *Tdc* promoter binds 3AF1-like nuclear factors from tobacco and *C. roseus*

As shown in Figs. 1A and 2A, fragments HD and TD actually bind two nuclear factors, one of which was identified as GT-1. The other complex migrated faster than the GT-1 complex. This complex was not competed out by 4II, indicating that it does not result from GT-1 binding. Fragment TD itself and the TD-containing HD fragment competed effectively for formation of the complex, whereas fragments HS and RH showed little competition. Fragments HT and DB did not compete for formation of the complex. In further competition experiments, the sequences 4F1 and 4F1m were used; they comprise four copies of the wild-type and mutant box VI sequence, respectively, from the pea *RbcS-3A* promoter (Lam et al. 1990). The box VI sequence was originally identified using in vitro footprinting studies and was shown to represent the binding site for the tobacco nuclear factor 3AF1. We observed that GT-1 also shows affinity for 4F1 (Fig. 3B and results not shown). To avoid interference with 3AF1 binding, all reactions included 4II in 25-fold molar excess, in order to saturate GT-1 binding activity in the nuclear extract. As shown in Fig. 3A for tobacco extract, a 100-fold or 50-fold molar excess of 4F1 competed less for the lower complex formed with probe TD than did the same amounts of competitor TD. The same amounts of the mutant 4F1m did not compete. These results clearly demonstrate that the lower TD complex is formed by the binding of nuclear factor 3AF1. In addition, the affinity of 3AF1 for TD appeared to be higher than for the other *Tdc* promoter fragments or for 4F1. Competition experiments using *C. roseus* nuclear extract gave identical results to those shown here for tobacco (Fig. 1B and results not shown).

The reverse experiment demonstrated that TD could compete for the 3AF1 complex formed with probe 4F1 (Fig. 3B). However, in contrast to the experiments with TD as probe, a 200-fold molar excess of unlabelled TD was not sufficient for complete competition of the 3AF1 complex, whereas the same amount of 4F1 competed out the complex almost completely. The mutant 4F1m sequence did not show competition in any of the reactions. The binding of 3AF1 to other *Tdc* promoter fragments was also studied using 4F1 as probe. Similar to the results with TD as probe, fragments HS, SR and RH also competed to some degree for the 3AF1 complex (Fig. 3B). The distribution of protein-binding activity in the *Tdc* promoter is summarized in Fig. 4.

At present the box VI sequence, AAATAGATAA-ATAAAAACATT, from the pea *RbcS-3A* promoter is

Table 1 Sequence comparison of experimentally identified GT-1 binding sites present in the promoters of various genes and derivation of a consensus sequence

box	Sequence[a]												Gene	Reference
box II	G	T	G	T	G	G	T	T	A	A	T	A	*RbcS-3A*	Green et al. (1987)
box II*	G	T	G	A	G	G	T	A	A	T	A	T	*RbcS-3A*	Green et al. (1987)
box III (rev)	A	G	A	T	A	G	T	G	A	A	A	T	*RbcS-3A*	Green et al. (1987)
box III *(rev)	A	G	A	G	T	G	T	A	A	A	T	T	*RbcS-3A*	Green et al. (1987)
box 1	A	A	A	A	G	G	T	T	A	A	A	A	*Cab-E*	Schindler and Cashmore (1990)
box 2r	G	C	A	C	C	G	T	T	A	A	A	C	*Cab-E*	Schindler and Cashmore (1990)
box 3	G	A	G	T	G	G	T	A	A	A	T	T	*Cab-E*	Schindler and Cashmore (1990)
box 4	A	G	C	A	G	G	T	A	A	A	A	C	*Cab-E*	Schindler and Cashmore (1990)
box 5	G	A	A	A	G	G	T	T	A	T	T	T	*Cab-E*	Schindler and Cashmore (1990)
box 6	A	T	G	G	G	G	T	T	A	A	A	A	*Cab-E*	Schindler and Cashmore (1990)
box 7	C	A	T	C	A	G	T	A	A	A	T	A	*Cab-E*	Schindler and Cashmore (1990)
Pcbox1	G	A	A	T	G	G	T	A	A	C	A	A	*PetE*	Fisscher et al (1994)
# 5	A	G	A	T	G	G	T	A	T	A	T	T	*Tdc*	This study
# 6	G	T	C	G	A	G	T	A	A	A	A	A	*Tdc*	This study
# 7	A	A	A	A	A	G	T	A	A	A	A	A	*Tdc*	This study
BN	A	T	T	T	T	G	T	A	A	T	T	A	*Str*	Pasquali et al. (1999)
BN rev	T	T	A	T	T	G	T	A	A	A	A	T	*Str*	Pasquali et al. (1999)
BN	A	A	A	C	C	G	A	T	A	A	A	A	*Str*	Pasquali et al. (1999)
	−5	−4	−3	−2	−1	−0	+0	+1	+2	+3	+4	+5		
Base distribution														
A	9	7	10	5	4	–	1	11	17	14	11	9		
G	7	4	4	3	9	18	–	1	–	–	–	–		
C	1	1	2	3	2	–	–	–	–	1	–	2		
T	1	6	2	7	3	–	17	6	1	3	7	7		
Consensus	G/A	N	N	N	N	G	T	A/T	A	A	A/T	A/T		
Previous consensus	G/T	A/T	G	T	G	G/A	A/T	A	A	A/T	G/A	A/T		Green et al. (1988)

[a] Gs that were shown to be critical for GT-1 binding by methylation interference analysis are *underlined*

the only published binding site for 3AF1. The *Tdc* promoter sequence from −575 to +198 does not contain sequences which are identical to the box VI sequence (see Fig. 5). Because the box VI sequence is quite AT rich, it has been suggested that 3AF1 represents a binding factor for AT-rich DNA (Terzaghi and Cashmore 1995). AT-rich sequences are present in fragment TD and in other *Tdc* promoter fragments with affinity for 3AF1, but it is unclear whether these particular sequences are actually involved in binding 3AF1.

Localization of GT-1 binding sites in the −238 to −99 *Tdc* promoter region by methylation interference analysis

Determination of the role of GT-1 in regulation of the *Tdc* promoter requires the precise identification of the nucleotides involved in GT-1 binding, to allow mutagenesis of GT-1 binding sites in chimaeric *Tdc-gusA* constructs. Localization of Gs that are critical for GT-1 binding to fragments HT and TD was accomplished using the dimethyl sulphate methylation interference technique. As shown in Fig. 6, Gs in the top strand of fragment HT at positions −212, −209 and −208 and of fragment TD at positions −158 and −139 were depleted in the bound fraction and enriched in the free fraction, thereby indicating the importance of these Gs in binding of nuclear GT-1. Methylation of Gs in the bottom strands did not interfere with GT-1 binding (results not shown).

The data obtained from these experiments are compatible with the distribution of GT-1 binding sites in the *Tdc* promoter. As shown in Fig. 5 and Table 1, G-212, G-209 and G-208 are present in putative binding site no. 5, which is located in HT. G-158 and G-139 are present in Nos. 6 and 7, both in TD. There is one putative GT-1 binding site (No. 8) in fragment TD in which no critical Gs were identified. Apparently, this sequence is not bound by GT-1.

Point mutations in fragments HT and TD decrease GT-1 binding

The methylation interference experiments showed the importance of five Gs in fragment HD which are involved in GT-1 binding. These Gs were mutated to Ts. The resulting fragments, HTm and TDm, were then tested in vitro using EMSAs. At first, extract titrations were performed to test the effect of the mutations on GT-1 binding. Incubation of the wild-type probe TD with increasing amounts of nuclear extract resulted in formation of increasing amounts of the upper complex (Fig. 7A), which was shown to represent GT-1 binding (Fig. 1B, D). The same amounts of extract tested with TDm as probe did not show detectable GT-1 binding. The lower complex, which was shown to contain nuclear factor 3AF1 (Fig. 3A), was not affected by the two

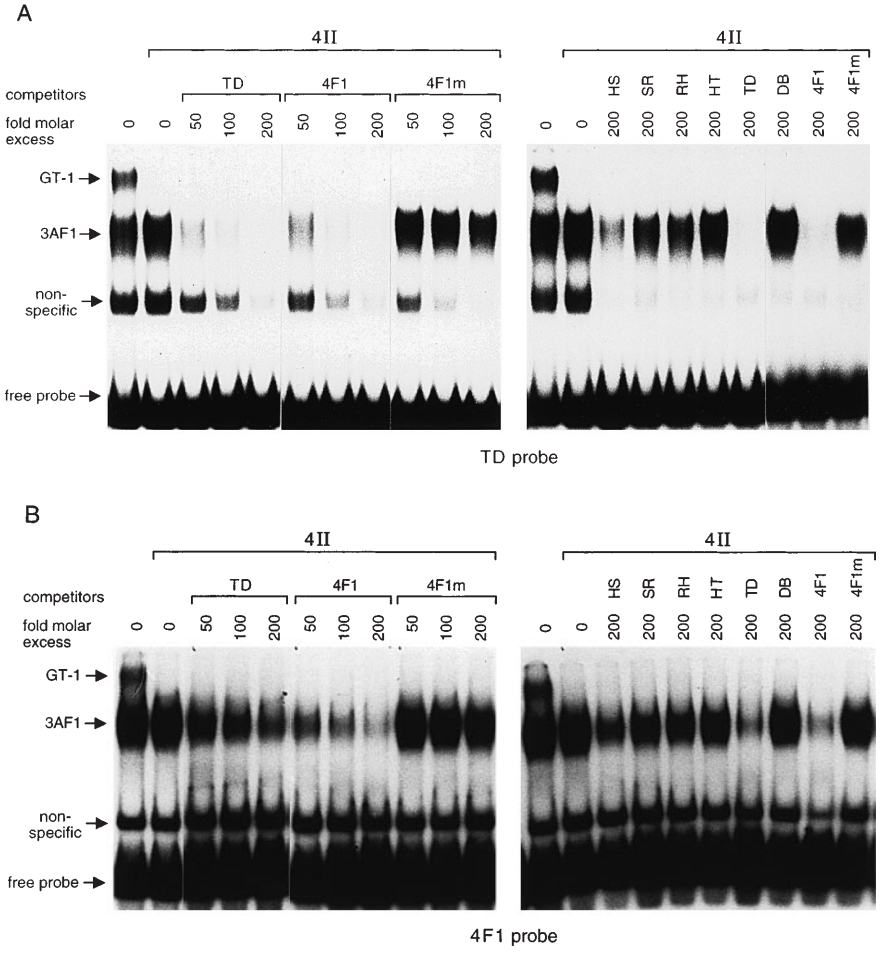

Fig. 3A, B Tobacco nuclear factor 3AF1 binds to several regions of the *Tdc* promoter. Probes TD **(A)** and 4F1 **(B)** were incubated with, respectively, 4 and 2 µg of tobacco nuclear extract. Competitor fragments were added in molar excess as indicated

mutations. In fact, 3AF1 appeared to bind slightly better to TDm. This may be due to the fact that more probe is available for 3AF1 binding in the absence of GT-1 binding. In addition, the TDm fragment is richer in A/T than TD, thereby increasing the affinity for the A/T-rich DNA binding factor 3AF1. Longer exposures showed no indication of GT-1 binding to probe TDm (results not shown). These results confirm the critical role of G-158 and G-139 in binding of GT-1.

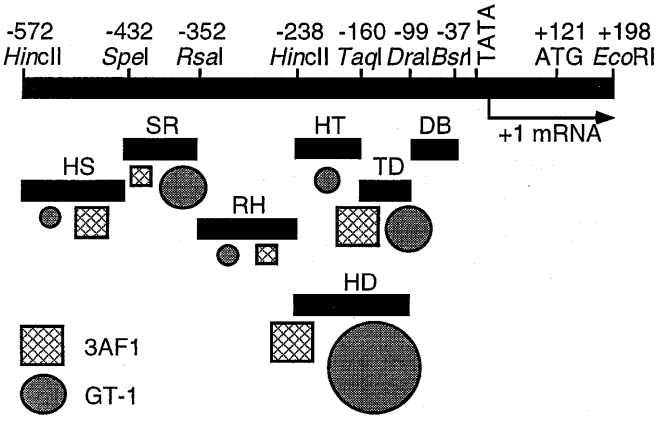

Fig. 4 Schematic overview of the multiple GT-1 and 3AF1 binding sites in the −572 to −37 region of the *Tdc* promoter. The restriction sites used to generate the competitor and probe fragments used in the EMSAs are indicated. The coordinates are given relative to the transcription start site at position +1 (Goddijn et al. 1994). The codes used for the promoter fragments refer to their flanking restriction sites. The relative affinities for GT-1 and 3AF1 are roughly indicated by the sizes of the symbols but these are not exactly to scale

The same experiment was also performed with probes HT and HTm. Incubation of probe HT with, respectively, 1, 2, 4 and 8 µg of nuclear extract resulted in increasing amounts of GT-1 binding (upper complex). Nuclear factor 3AF1 (lower complex), was found to have a very low affinity for fragment HT (Fig. 3B). As shown in Fig. 7B, the three point mutations in probe HTm do not completely prevent GT-1 binding. Comparison of the amounts of GT-1 complex formed with the probes HT and HTm shows that 8 µg of nuclear extract gives the same amount of GT-1 binding to probe HTm as the combination of 2 µg of nuclear extract and probe HT. Therefore, the mutations substantially decrease the affinity of HT for GT-1.

The effects of the mutations in HTm, TDm and HDm on GT-1 binding were also studied in EMSA competition experiments using TD as a probe (Fig. 8). As in the competition reactions presented in Fig. 1C and 2B, HD and 4II competed to the same extent,

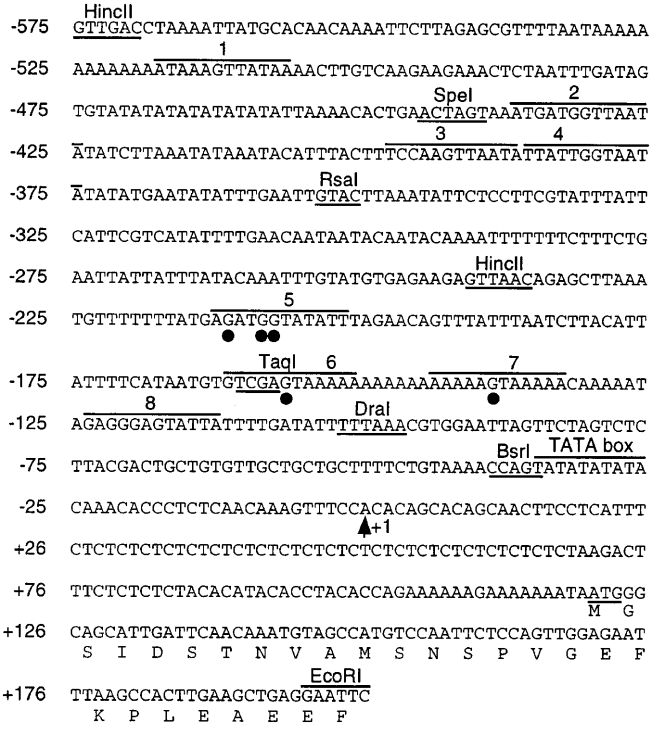

Fig. 5 *Tdc* promoter sequence from −575 to +198 bp. The putative TATA box (−34 to −25), mRNA transcription start site +1 and the *Tdc* translation start codon at +121 are indicated (Goddijn et al. 1994). The restriction sites used to generate fragments for constructs or EMSAs are underlined and the Gs which are critical for GT-1 binding are indicated with *dots*. The numbers 1–8 refer to the *overlined* sequences which show some similarity to the consensus GT-1 binding site (Green et al. 1988). The deduced amino acid sequence of the N-terminal part of the TDC enzyme is indicated in one-letter code. The sequence of the *Tdc* gene has been deposited in the DDBJ/EMBL/GenBank database under Accession No. X67662

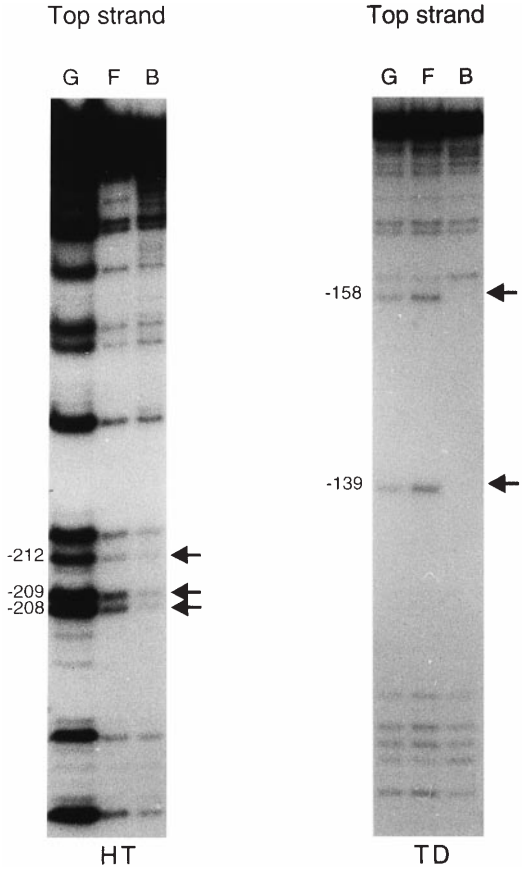

Fig. 6 Identification of Gs involved in GT-1 binding to the top strands of *Tdc* fragments HT and TD using methylation interference assays. The positions of Gs (lanes G) were determined by the G-strand reaction according to Green et al. (1991). Lanes F and B show the reaction products after chemical cleavage of the free (F) and bound (B) EMSA fractions

whereas TD and HT both competed less efficiently. The mutant 4IIm sequence did not compete at all. The affinity of HDm for GT-1 is comparable to that of HT and is thus far less than the affinity of HD or 4II for the protein. A 100-fold excess of TD completely competed for GT-1 binding whereas the same amount of TDm did not. Fragment HT, which interacts weakly with GT-1 (Fig. 2A-C), slightly reduces GT-1 binding when present at 100-fold molar excess, whereas the mutant HTm gave no detectable competition.

In conclusion, the results clearly show that the mutations in the *Tdc* promoter fragments HT and TD are highly effective in reducing binding of GT-1, without affecting 3AF1 binding. Thus, reporter gene constructs containing these mutations are suitable for examining the role of GT-1 in *Tdc* promoter regulation in vivo.

Mutations in GT-1 binding sites in the *Tdc* promoter decrease UV light induction

To address the question whether GT-1 interacts in vivo with its binding sites in the HD segment of the *Tdc* promoter and determines the expression of the *Tdc* promoter, several mutant *Tdc-gusA* constructs, presented in Fig. 9B, were analysed in transgenic tobacco. The mutant construct Δ278m contained, respectively, three and two point mutations in fragments HT (−238 to −160) and TD (−160 and −99). Otherwise it is completely equivalent to construct Δ278. Deletion of the HT-containing region (−278 to −160) resulted in construct Δ160. Construct Δ160 contains two GT-1 binding sites, both of which are located in fragment TD. These binding sites are mutated in construct Δ160m. The mutant TDm fragment was also tested as a tetramer on the *Tdc* Δ37 truncated promoter (construct 4TDm-37) and compared with the wild-type construct 4TD-37. Each construct was analysed by measuring GUS activities in 9–16 independent tissue-cultured transgenic plants. The results are presented in scatter diagrams in which each symbol represents the GUS activity of a single plant. The significances of differences in GUS levels between wild type and mutant or between different 5′ deletion constructs were calculated by using the Wilcoxon matched pairs-signed rank test.

As shown in Fig. 9A, plants harbouring constructs Δ278 and Δ160 show basal expression levels which do

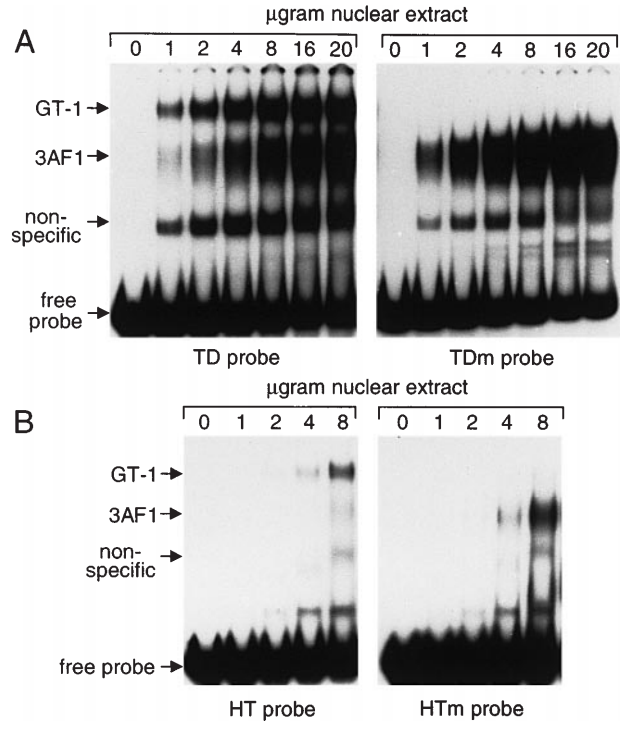

Fig. 7A, B Point mutations in fragments TD and HT eliminate or reduce GT-1 binding and do not reduce binding of 3AF1. Binding reactions using probes TD and TDm **(A)** or probes HT and HTm **(B)** contained the indicated amounts of tobacco nuclear extract

not differ significantly, as indicated by the high p value ($p = 0.796$). Omission of region TD from construct Δ160, resulting in construct Δ99, reduces expression significantly to almost zero ($p = 0.001$), indicating the importance of TD as an enhancer. This is confirmed by comparing constructs Δ37 and 4TD-37. Construct Δ37 contains only the TATA box and has a low expression level. Addition of TD as a tetramer increased expression by over 13.6-fold. Mutagenesis of GT-1 binding sites in constructs Δ278m, Δ160m and 4TDm-37 did not result in significantly altered basal expression levels (p values for wild type versus mutant are 0.796, 0.203 and 0.260 respectively).

To determine whether the mutations in GT-1 binding sites affect expression mediated by external signals, we tested the constructs with fungal elicitor and UV light. No significant differences in elicitor responsiveness were observed between the wild-type and mutant constructs (results not shown). In contrast, the mutations did have significant effects on UV-induced expression. As shown in Fig. 9A, mutations in the GT-1 binding sites in Δ278m decreased the UV-induced expression levels considerably. When compared to basal expression the p value of a Wilcoxon matched pairs-signed rank test of constructs Δ278 versus Δ278m drops from 0.796 (basal) to 0.089 (UV), indicating that the difference between the UV-induced expression levels of wild type and mutant is significant. The fact that UV inducibility of construct Δ278m is not completely lost is likely to be due to the redundancy of UV light-inducible elements in the *Tdc* promoter. For instance, construct Δ99 and Δ37 are also inducible by UV, and the region between −99 and −37 was shown to contain an UV-inducible enhancer (our unpublished results). On comparing constructs Δ160 and Δ160m or 4TD-37 and 4TDm-37 it is again observed that the mutation of GT-1 binding sites renders the constructs less responsive to UV light (Fig. 9A). For these constructs the p values corresponding to basal and UV-induced levels are 0.203 versus 0.123 and 0.260 versus 0.027, respectively. Since a reproducible effect of mutations in GT-1 binding sites on UV light-induced expression levels is observed with all constructs analysed, we conclude that GT-1 binding contributes to UV-mediated gene expression. As a role for GT-1 in regulation by normal white light has also been postulated (Lam and Chua 1990), we also analysed the expression of the wild-type and mutant *Tdc* promoter constructs under light and dark conditions. Neither in developing seedlings nor in mature plants could any differences be detected between *Tdc* expression in the light and in the dark (results not shown).

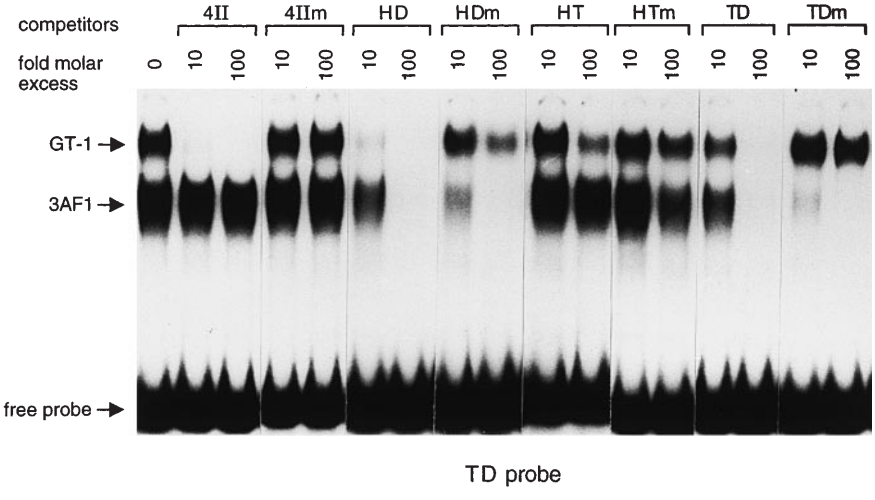

Fig. 8 Mutated *Tdc* promoter fragments compete less efficiently for GT-1 binding to fragment TD. Probe TD was incubated with 4 µg of tobacco nuclear extract. Competitor fragments were added in molar excess as indicated

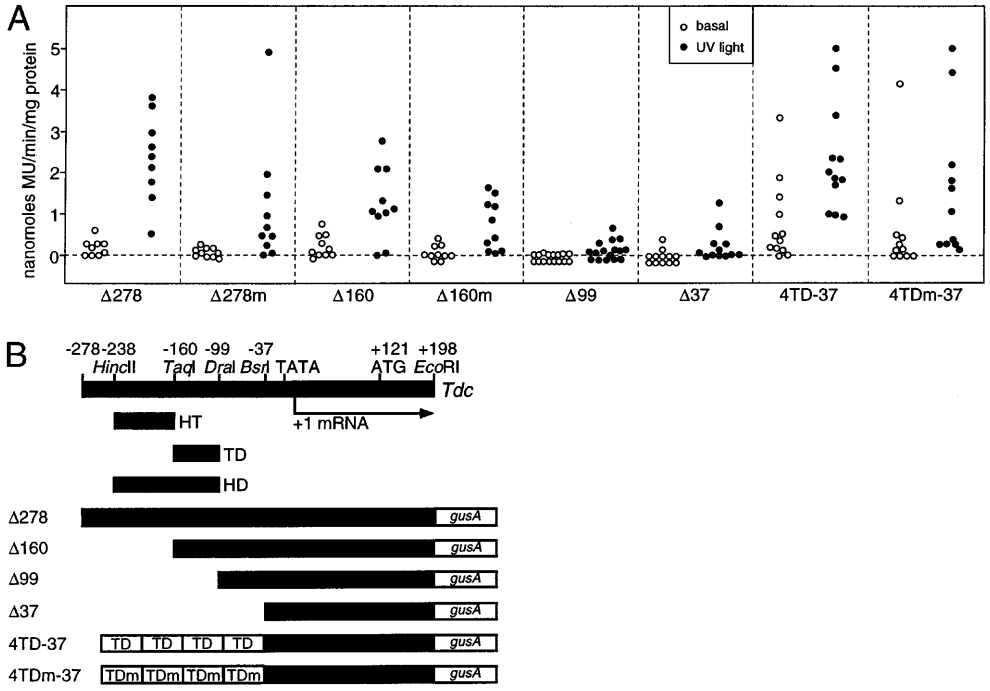

Fig. 9A, B Elimination of GT-1 binding sites reduces UV-responsive *Tdc* expression in transgenic tobacco plants. **A** GUS activities were measured in protein extracts prepared from water- (basal) or UV light-treated leaf discs and are represented by the *open* and *closed circles*, respectively. For each construct the GUS activities of protein extracts originating from 9–16 independent transgenic plants are presented. Symbols below the zero level indicate that GUS activities were below the detection limit. **B** Schematic representation of the *Tdc* promoter from –278 to +198. The restriction sites used to generate the competitor or probe fragments for EMSAs, or to assemble translational fusion constructs with the *gusA* reporter gene, are indicated. Tetramers of TD and TDm were cloned in front of the *Tdc* Δ37 truncated promoter, shown at the bottom. The coordinates of the *Tdc* gene are given relative to the transcription start site at position +1. The codes used for the promoter fragments refer to their respective flanking restriction sites

Discussion

The tryptophan decarboxylase enzyme encoded by the *Tdc* gene catalyses a key step in the biosynthesis of TIAs, which have putative defence-related functions in *C. roseus*. Previous studies of the expression of *Tdc-gusA* fusion constructs in transgenic tobacco showed that regulatory elements required for basal and stress-induced expression are located in the −160 to −99 and −99 to −37 promoter regions, respectively (Goddijn et al. 1994; Ouwerkerk and Memelink 1999a). As a first step toward identifying nuclear factors involved in *Tdc* expression, interactions between promoter fragments and nuclear proteins extracted from tobacco leaves and suspension-cultured *C. roseus* cells were studied. Two distinct nuclear factors were found to bind multiple sites in the −572 to −99 region. Based on their binding characteristics, abundance and electrophoretic mobility, these factors could be identified as the previously described factors GT-1 (Green et al. 1987) and 3AF1 (Lam et al. 1990). Furthermore, antibodies against a cloned GT-1b protein from tobacco recognized the tobacco nuclear protein that formed the GT-1-like complex with *Tdc* promoter fragment HD. GT-1 and 3AF1 showed the same binding characteristics whether extracted from tobacco leaf or from *C. roseus* suspension-cultured cells. This indicates that these factors are not specific for either plant species or cell type.

A schematic overview of the binding of GT-1 and 3AF1 to the *Tdc* promoter is presented in Fig. 4. Among the *Tdc* promoter fragments tested, HD exhibited the highest affinity for GT-1. Testing of its subfragments showed that the affinities of TD and HT for GT-1 are, respectively, about 20- and 200-fold lower than that of the complete fragment HD. This suggests that the affinity of HD for GT-1 is not simply the sum of the affinities of its subfragments, but reflects a cooperative interaction between GT-1 molecules. Similar results have been obtained with box II, which binds GT-1 cooperatively in combination with additional box II copies or with other GT-1 binding sites in the *RbcS-3A* promoter (Green et al. 1987, 1988).

Methylation interference analysis was used to localize the Gs to which GT-1 binds in the HD subfragments HT and TD. As expected, the critical Gs are located in sequences which have similarity to GT-1 binding sites. Inspection of all GT-1 binding sites identified thus far using methylation interference, by us and by others (Table 1), revealed new information about the binding site preference of GT-1. As indicated, the sequences of defined GT elements show much variation when compared to the pea *RbcS-3A* boxII sequence which was among the first set of GT-1 binding sites identified (Green et al. 1988). We noticed that if multiple critical Gs are present in a binding site, the G situated at the 3′

end of the sequence is always followed by a T. Similarly, when only one G is found to be critical for GT-1 binding, the next nucleotide is also a T. The *Str* promoter is the sole known exception to this rule (Pasquali et al. 1999), but in 17 other cases a GT core was found to be present. We designated these two central nucleotides G and T as −0 and +0, respectively. The nucleotide at +1 is preferentially an A, less often a T. Positions +3 and +4 are also preferentially As, and only in a few cases are Ts present. From the list in Table 1 it is clear that Ts are never present in both positions +2 and +3. This combination is present in putative binding site No. 4 in the *Tdc* promoter (Fig. 5). This site was not identified by methylation interference and is apparently not recognized by GT-1. Based on our observations a new consensus GT-1 binding site is presented: (G/A)NNN(G/A)G T(A/T)AA(A/T)(A/T) (Table 1). This sequence is an improvement on a previously proposed consensus binding site (G/T)(A/T)GTG(G/A) (A/T)AA(A/T)(G/A)(A/T) (Green et al. 1988), which failed to recognize the conserved GT core sequence.

The second factor that binds to the *Tdc* promoter, 3AF1, bound with different degrees of affinity to fragments HS, RH and TD. Among these fragments, TD exhibited the highest affinity for 3AF1. Using TD as a probe it was found that TD was more effective in competing for the 3AF1 complex than a tetramer (4F1) of box VI from the pea *RbcS-3A* promoter. The reverse experiment with 4F1 as probe showed that 4F1 was more effective in competing for the 3AF1 complex than TD. One explanation for this apparent contradiction is that multiple forms of 3AF1 exist with different affinities for different DNA sequences. A cDNA clone has been isolated from tobacco, which encodes a zinc-finger protein that binds specifically to 4F1 (Lam et al. 1990). Northern hybridization analysis with the 3AF1 cDNA clone revealed multiple transcript sizes, supporting the existence of a family of 3AF1-like factors. Attempts were made to identify 3AF1 binding sites in the *Tdc* promoter using methylation interference, but these were unsuccessful (results not shown). This could mean that methylation of Gs does not interfere with 3AF1 binding or that no Gs are involved in 3AF1 binding, which would support the suggestion that 3AF1 binds AT-rich DNA (Terzaghi and Cashmore 1995).

Analysis of the effect on gene expression of mutations that reduce binding has provided evidence that GT-1 and 3AF1 are transcription factors that regulate the expression of the pea *RbcS-3A* gene in tobacco (Green et al. 1988; Kuhlemeier et al. 1988; Lam and Chua 1990). Our observation that the affinities of GT-1 and 3AF1 for the −160 to −99 region equal or even exceed those for the respective binding sites in the pea *RbcS-3A* promoter argues strongly for a functional role of GT-1 and 3AF1 in regulation of the *Tdc* gene, via interaction with the TD region. The fact that deletion of the region of the *Tdc* promoter from −278 to −112 considerably decreases the expression level provides circumstantial evidence for an important role of the −160 to −99 (TD) region. More direct evidence comes from a set of loss- and gain-of-function analyses that demonstrated that TD is important for basal expression (Ouwerkerk and Memelink 1999a). We were able to identify Gs involved in GT-1 binding within the *Tdc* promoter. Mutagenesis of these Gs considerably reduced or even eliminated binding in vitro. To our surprise the same mutations, when analysed in reporter gene constructs in transgenic tobacco, did not affect basal expression. Also no differences were found in expression patterns during development from seedling to mature plant, either in the light or in the dark. However, induction of the *Tdc* promoter by UV light was shown to be reduced by the mutations. We checked whether UV light influenced the binding of nuclear GT-1 to *Tdc* promoter fragments in vitro, by using nuclear extracts prepared from UV-irradiated tobacco leaves, but no differences were found (results not shown). This suggests that UV regulation via GT-1 might be dependent on other factors, which interact with GT-1 and thereby regulate the effect of GT-1 on expression. UV light is widely considered to represent a stress factor for plants, since it can have a negative impact on various plant processes, such as growth and reproduction. Most probably, such effects are the result of damage to proteins, membranes or to DNA (Stapleton 1992). Plants protect themselves against UV light by producing UV-absorbing secondary metabolites such as flavonoids (Dixon and Paiva 1995). It has also been proposed that TIAs protect against the effects of UV light (Hartmann 1991). In agreement with this hypothesis, the formation of several TIAs is induced in *C. roseus* leaves after irradiation with UV light (Hirata et al. 1992), and the TIA biosynthetic genes *Tdc*, *Str* (unpublished results) and *Cpr* (Meijer 1993) are all induced by UV light. Most studies on UV light-induced gene expression have focused on flavonoid biosynthetic genes, and resulted in the identification of several *cis*-regulatory sequences present in promoters (Schulze-Lefert et al. 1989; Hartmann et al. 1998). GT-1 has not previously been shown to be involved in responsiveness to UV light.

Our findings contrast with reports on the role of GT-1 in regulation of the pea *RbcS-3A* gene. Expression of a −175 deletion construct of the *RbcS-3A* gene seemed to be determined by GT-1, since mutation of GT-1 binding sites in this construct severely reduced expression in the light (Kuhlemeier et al. 1988). Based on this result and the observation that a tetramer of the *RbcS-3A* GT-1 binding site conferred light-specific expression on a truncated CaMV 35S promoter (Lam and Chua 1990), it was proposed that GT-1 represents a specific regulator that is activated by light. Furthermore, GT-1 is thought to function as a light-regulatory transcriptional activator of a number of other light-regulated genes (Green et al. 1987, 1988; Schindler and Cashmore 1990; Fisscher et al. 1994; Teakle and Kay 1995; Tjaden et al. 1995). On the other hand, GT-1 does not appear to be involved in regulation of the *Tdc* promoter by light, as GT-1 binding-site mutations did not affect basal expression of our *Tdc* promoter constructs under various light/dark con-

ditions. Moreover, our results and those of others (Villain et al. 1994) demonstrate that the function of GT-1 is not restricted to light-dependent activation. GT-1 appears to contribute to activation of *Tdc* by UV light, and was shown to function as a repressor in the light and in darkness in the case of the spinach *Rps1* promoter (Villain et al. 1994). It is possible that the role of GT-1 in regulating expression of a specific gene is determined by interactions with other *trans*-acting factors. In the case of the *RbcS-3A* promoter possible interacting partners are the nuclear factors 3AF1 (Lam et al. 1990), 3AF3 and 3AF5 (Sarokin and Chua 1992), which also bind close to the GT-1 binding sites in the −175 *RbcS-3A* promoter. In the case of the *Tdc* promoter the interacting partner could be 3AF1, which binds with high affinity to the same fragments as GT-1 does.

Besides the *Tdc* promoter, other defence-related gene promoters have also been reported to bind GT-1. A promoter fragment of the bean chalcone synthase gene *Chs15* was shown to contain three different binding sites for nuclear factor SBF-1, which has the same binding specificity as GT-1 and is likely to be identical to it (Lawton et al. 1991). GT-1 was also shown to interact with the promoter of the pathogenesis-related gene *PR-1a* (Buchel et al. 1996). Furthermore, GT-1 interacts with two other genes, *Str* and *Cpr*, that operate in the *C. roseus* TIA pathway (Lopes-Cardoso et al. 1997; Pasquali et al. 1999). Thus, the available binding studies suggest that GT-1 represents a regulator not only of light-regulated, but also of defence-related genes. Again, the specific role of GT-1 in regulation of different defence-related genes seems to be dependent on interactions with other factors, as GT-1 binding is correlated with silencing in the case of the *Chs* and *PR-1a* genes, but binds to strong enhancers in the *Str*, *Cpr* and *Tdc* genes.

Clearly, a consistent overall picture of the significance of GT-1 binding sites in different gene promoters is lacking. There is a need for promoter studies that establish whether in vitro GT-1 binding sites play any role in expression of the corresponding gene in vivo, and if so, whether they act as transcriptional silencers or enhancers. For the *Tdc* promoter we succeeded in identifying GT-1 binding sites in vitro and in correlating these sites with UV-enhanced expression. The question still remains which *trans*-acting factors are responsible for the strong basal activity of the *Tdc* promoter. Another obvious candidate is 3AF1, which binds with high affinity to fragment TD. Furthermore, it is perfectly possible that still other *trans*-acting factors exist, which were not detected in EMSAs, but which have significant functions in vivo.

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