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Modulation of GT-1 DNA-binding activity by calcium-dependent phosphorylation

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Abstract

The analysis of pea *rbcS-3A* promoter sequence showed that BoxII was necessary for the control of *rbcS-3A* gene expression by light. GT-1, a DNA-binding protein that interacts with BoxII *in vitro*, is a good candidate for being a light-modulated molecular switch controlling gene expression. However, the relationship between GT-1 activity and light-responsive gene activation still remains hypothetical. Because no marked *de novo* synthesis was detected after light treatment, light may induce post-translational modifications of GT-1 such as phosphorylation or dephosphorylation. Here, we show that recombinant GT-1 (hGT-1) of *Arabidopsis* can be phosphorylated by various mammalian kinase activities *in vitro*. Whereas phosphorylation by casein kinase II had no apparent effect on hGT-1 DNA binding, phosphorylation by calcium/calmodulin kinase II (CaMKII) increased the binding activity 10–20-fold. Mass spectrometry analyses of the phosphorylated hGT-1 showed that amongst the 6 potential phosphorylatable residues (T86, T133, S175, T179, S198 and T278), only T133 and S198 are heavily modified. Analyses of mutants altered at T86, T133, S175, T179, S198 and T278 demonstrated that phosphorylation of T133 can account for most of the stimulation of DNA-binding activity by CaMKII, indicating that this residue plays an important role in hGT-1/BoxII interaction. We further showed that nuclear GT-1 DNA-binding activity to BoxII was reduced by treatment with calf intestine phosphatase in extracts prepared from light-grown plants but not from etiolated plants. Taken together, our results suggest that GT-1 may act as a molecular switch modulated by calcium-dependent phosphorylation and dephosphorylation in response to light signals.

Introduction

The early transcriptional control of nuclear gene expression by light was first demonstrated by 'run-on' assays using nuclei from light-treated and dark-adapted plants and monitoring the expression of genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) and a chlorophyll *a,b*-binding protein (*cab*) from the light-harvesting complex II (Gallagher and Ellis, 1982). Photoreceptors involved in light perception have been identified;

they include at least three families of photoreceptors that detect different wavelenghts: the phytochromes, which detect red and far-red light, the blue light photoreceptors, and the ultraviolet light photoreceptors (Furuya, 1993; Lin *et al.*, 1996; Kim, 1998). Of these, the best characterized are the phytochromes (Furuya, 1993). The understanding of light signal transduction has benefited from an experimental system that employs microinjection to deliver putative signaling intermediates into single cells of the tomato *aurea* mutant that has greatly reduced levels of phytochromes (PHY A) (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994; Wu *et al.*, 1996). Using this system, it was shown that both *rbcS* and *cab* genes are activated by PHY A via a cytosolic Ca^{2+} increase, whereas PHY A activation of other light-responsive genes such as *chs* (encoding chalcone synthase) are mediated by cGMP (Bowler *et al.*, 1994).

Light-responsive expression of the pea *rbcS-3A* gene in transgenic tobacco led to the identification of *cis*-acting elements that interact with a number of *trans* factors (Kuhlemeier *et al.*, 1987, 1988; Gilmartin *et al.*, 1990). GT-1 which binds to six binding sites in the upstream region of *rbcS-3A*, was identified as one such factor (Green *et al.*, 1987, 1988). Although GT-1 DNA-binding sites are relaxed (i.e., the loose GT consensus $^{G}_{T}{}^{A}_{T}$GTGPu$^{A}_{T}$AA$^{A}_{T}$Pu$^{A}_{T}$ and the GATA motif), *in vitro* experiments indicated highest specificity for the core sequence GGTTAA of the BoxII element (GTGTGGTTAATATG) in the *rbcS-3A* promoter (Hiratsuka *et al.*, 1994).

Studies of reporter genes fused downstream of BoxII multimers demonstrated that BoxII could act as a light-responsive element (LRE) (Lam and Chua, 1990; Puente *et al.*, 1996; Chattopadhyay *et al.*, 1998). A synthetic tetramer of *rbcS-3A* BoxII fused to the −90 deleted cauliflower mosaic virus 35S promoter was sufficient to confer light-responsive activation of the β-glucuronidase (GUS) reporter gene (Lam and Chua, 1990). Using microinjection into single cells of the tomato *aurea* mutant, Wu *et al.* (1996) showed that 11 copies of *rbcS-3A* BoxII linked 5′ to the −90 35S promoter and fused to the GUS reporter gene (11BoxII::GUS), was activated by co-injection of oat phytochrome A or GTPγS, an activator of heterotrimeric G protein. In addition, calcium, but not cGMP, induced 11BoxII::GUS expression. BoxII was therefore identified as a LRE being activated by phytochrome A via calcium.

The relationship between GT-1 *trans* activity and light activation of *rbcS* genes is still hypothetical. In gel-retardation experiments, Green *et al.* (1987) showed that nuclear extracts from dark-adapted plants exhibited a DNA-binding activity with the target site sequence specificity of GT-1. This result was further confirmed in footprinting experiments using the −330 to −50 fragment from *rbcS-3A* with extracts from light-grown and dark-adapted pea seedlings (Green *et al.*, 1988). Because no marked *de novo* synthesis of GT-1 was detected after light treatment, it was proposed that light may induce post-translational modifications of GT-1. In addition, productive and non-productive binding of GT-1 and BoxII might occur (Green *et al.*, 1988).

GT-1-like DNA-binding proteins have been reported for various plant species, such as tobacco (Green *et al.*, 1988), bean (Lawton *et al.*, 1991), spinach (Oroczo and Ogren, 1993), parsley (Harter *et al.*, 1994) and *Arabidopsis* (Kuhn *et al.*, 1993). Because of its possible involvement in the regulation of light-responsive genes, cDNA clones encoding GT-1 protein have been characterized from tobacco (Gilmartin *et al.*, 1990) and *Arabidopsis* (Hiratsuka *et al.*, 1994), and cDNA clones encoding other GT-1-like proteins (GT-2) have been isolated from rice (Dehesh *et al.*, 1990) and *Arabidopsis* (Kuhn *et al.*, 1993).

Hiratsuka *et al.* (1994) and Lam (1995) dissected the molecular structure of GT-1 from *Arabidopsis* and tobacco, respectively. Both GT-1 factors contain two functional domains: (1) a predicted tri(tetra)helix motif necessary for DNA-binding, and (2) a C-terminal protein-protein interaction domain. *Arabidopsis* GT-1 bound to target DNA as a dimer (Hiratsuka *et al.*, 1994), whereas size exclusion chromatography analyses indicated that tobacco GT-1 formed tetramers in solution (Lam, 1995). Tobacco GT-1 oligomerization was shown to be independent of DNA-binding (Lam, 1995).

Figure 1 shows an alignment of *Arabidopsis* and tobacco GT-1 sequences. Together, serine and threonine represent 13% of the total amino acids from *Arabidopsis* GT-1 and 13.7% of the total amino acids from tobacco GT-1. The positions of these residues were remarkably conserved between the tobacco and *Arabidopsis* proteins. In some cases, a serine is substituted for threonine at the same position in the two proteins (indicated by § in Figure 1), suggesting that a phosphorylatable residue is critical in that position. Six serine and threonine residues belong to the basic-X-X-serine/threonine consensus motif (T86, T133, S175 and T278 in the R-X-X-S/T con-

sensus domain, and T179, S198 in the K-X-X-S/T domain) which is a substrate for mammalian multifunctional calcium/calmodulin kinase II (CaMKII) and calcium-dependent proteine kinases from plants (CDPKs). In addition, 6 residues belong to the casein kinase II (CKII) substrate consensus motif (Pearson and Kemp, 1991) (see Figure 1). Interestingly, all GT-1 and GT-2 factors cloned so far contain putative targets for CaMKII or CDPKs, i.e. the basic-X-X-serine/thereonine domains: 5 in tobacco GT-1 (Figure 1), 8 in *Arabidopsis* GT-2, and 10 in rice GT-2.

In the present study, we used a purified recombinant *Arabidopsis* GT-1 protein to investigate the effects of GT-1 phosphorylation by various kinases at the level of BoxII binding *in vitro*. We showed that phosphorylation of *Arabidopsis* GT-1 on specific residues of basic-X-X-serine/threonine domains could regulate GT-1 binding to BoxII. We further provided evidence that GT-1 DNA-binding activity in light-grown plants is dependent upon GT-1 phosphorylation *in vivo*. Our results suggest a mechanism by which GT-1 could function as a molecular switch responding to light and probably other inputs by integrated phosphorylation and/or dephosphorylation events.

Materials and methods

Chemicals

γ^{32}P-ATP, α^{32}P-dATP and α^{32}P-dCTP (specific activity 3000 Ci/mmol) were obtained from Amersham; unlabelled ATP and dNTPs, poly(dI:dG) and calf intestine alkaline phosphatase (CIP) from Pharmacia LKB Biotechnology; bovine calmodulin, casein kinase II (CKII), isopropylthiogalactoside (IPTG) and lysozyme from Sigma, and KN-92 and KN-93 from Molecular Probe. All other chemicals were analytical grade.

General molecular biology techniques

DNA gel blot hybridizations as well as other standard molecular cloning techniques were performed as described previously (Sambrook *et al.*, 1989). DNA sequencing was carried out using a Sequenase DNA sequencing kit (US Biochemical) according to the manufacturer's instructions. DNA and amino acid sequence data were processed using a Macintosh DNASIS program software package (Hitachi Software Engineering Co., San Bruno, CA).

Plant nuclear extract preparation

Nuclear extracts were prepared from light-grown pea as described (Green *et al.*, 1987). Nuclear extracts were prepared from etiolated pea seedlings under special conditions. All steps in the nuclei purification were carried out at 2 °C under green safe lights. Three 5 g plumules, harvested from 7-day old etiolated pea seedlings, were immersed in cold diethyl ether for 5 min and then air-dried. Homogenization was carried out in 30 ml of 1 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, 10 mM HEPES pH 7. After filtration through 3 layers of Miracloth, 1 layer each of 88 μm and 30 μm nylon mesh, the homogenate was centrifuged at 1900 $\times$ g for 10 min. The pellet was resuspended in 5 ml of homogenization buffer and centrifuged (7800 $\times$ g, 30 min) over a discontinuous Percoll gradient containing 5 ml each of 50% and 25% Percoll in homogenization buffer. Nuclei were collected from the interface of the two layers, washed twice in homogenization buffer to remove residual Percoll, and resuspended in 20% glycerol, 6 mM $MgSO_4$, 1 mM DTT, 60 mM HEPES pH 7.

Expression of hGT-1 in E. coli *and purification of the recombinant protein*

The coding region of *Arabidopsis* GT-1 (Hiratsuka *et al.*, 1994) was cloned into the *Nde*I/*Xho*I site of the polylinker of pET-15b (Novagen). Because of a hexahistidine encoding domain adjacent to the 5′ end of the cloning site, the GT-1 was expressed as a fusion protein with 6 extra histidine residues at the N-terminus. This recombinant protein was referred to as hGT-1. A culture of the *Escherichia coli* strain carrying the expression construct (BL21) was grown overnight, diluted 1:5 in Luria broth medium, and grown for 3 h at 30 °C. IPTG was added to a final concentration of 0.4 mM, and culture growth was continued for 2 h. Bacteria (100 ml) were harvested by centrifugation, and the pellet was resuspended in 4 ml of ice-cold buffer A (0.5 M NaCl, 20 mM Tris-HCl pH 8) containing 5 mM imidazole. After a brief sonication, the cleared lysate was centrifuged at 39 000 $\times$ g for 20 min, and the supernatant was loaded onto an activated Sepharose 6B resin (Novagen) charged with 50 mM $NiSO_4$, and extensively washed with water. The column was washed with 10 volumes of binding buffer and 6 volumes of 60 mM imidazole (in buffer A), and was eluted with 1 M imidazole (in buffer A). The eluted fractions were analyzed for the presence of

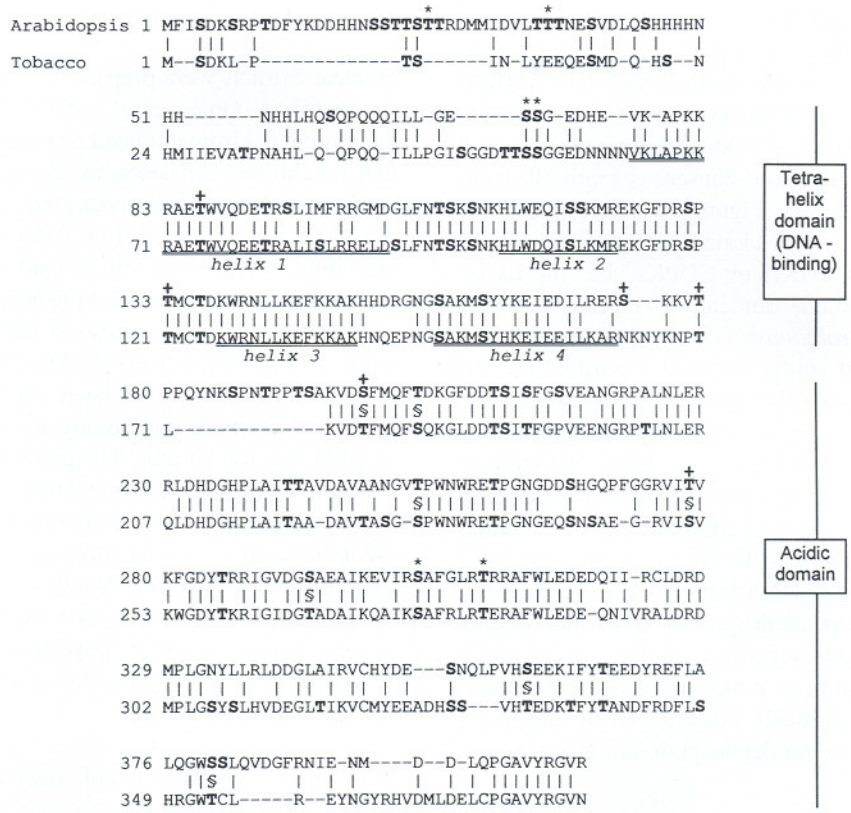

Figure 1. Tobacco and *Arabidopsis* GT-1 share evolutionarily conserved consensus motifs for phosphorylation by CKII and CaMKII-like kinases. Alignment of GT-1 sequences from *Arabidopsis* (EMBL accession number L36806; Hiratsuka *et al.*, 1994) and tobacco (EMBL accession number M93436; Perisic and Lam, 1992). Serine and threonine are represented using bold characters (**S** and **T**). Identical amino acids are indicated by (|); serines in place of threonines in the reciprocal sequence are indicated by (§). The putative oligomerization region of GT-1 is localized in the C-terminal acidic region (with high aspartic acid, glutamic acid, asparagine and glutamine contents). The putative α-helices (in a tri(tetra)helix domain involved in DNA-binding) are underlined. Serine and threonine within a putative substrate motif for CKII (S/T-X-X-E/D and S/T-E-E/D) and CaMKII (K/R-X-X-T/S) are indicated by a ($\star$) and a (+) respectively. In this study, the effects of specific point mutations were investigated and these mutations are indicated by (+).

a 51 kDa polypeptide by SDS-PAGE; those containing this polypeptide were pooled and dialyzed against 50 mM Tris-HCl, 5 mM EDTA pH 8.

Recombinant protein storage

Purified recombinant hGT-1 was unstable. Freezing and thawing resulted in protein precipitation. This precipitate could be re-dissolved by raising the pH to 9.2 with diluted NaOH; however, under these conditions no DNA-binding activity could be measured. Therefore recombinant hGT-1 protein was stored at −20 °C at a final concentration of 4 mg/ml in glycerol (50%), 20 mM Tris-HCl, 5 mM EDTA pH 8, and diluted in any reaction buffer so that the final glycerol concentration did not exceed 10%. Under these conditions 90% of the original DNA-binding activity of GT-1 was still detectable after 3 weeks of storage.

Site-directed mutagenesis

In vitro mutagenesis of GT-1 cDNA was performed using a double-stranded, site-directed mutagenesis kit (Stratagene), allowing the site-specific mutation of the pET-15b plasmid containing the GT-1 coding sequence. A selection primer (CTCTGCTAATCCTGTTACCAGTCGCGACTGCCAGTGGCG) was designed to change the non-essential unique *Alw*NI restriction site from pET-15b to a *Nru*I site. Primers encoding for specific mutations in GT-1 sequence were designed to change specifc threonine (T) or serine (S) residues to alanine (A) or aspartic acid (D) residues: T86A (CGAGCGGAGGCATGGGTTCAA-

GACG), T86D (CGAGCGGAGGACTGGGTTCAAGACG), T133A (GGGTTTGATCGATCTCCGGCTATGTGTACTG), T133D (GGGTTTGATCGATCTCCGGATATGTGTACTG), S175A (CTTAGAGAGAGGGCCAAAAAAGTGACACCACC), S175D (CTTAGAGAGAGGGACAAAAAAGTGACACCACC), T179A (GAGAGGAGCAAAAAAGTGGCACCACCACAG), T179D (GAGAGGAGCAAAAAAGTGGACCCACCACAG), S198A (CAGCCAAAGTTGATGCCTTTATGCAATTTACTG), S198D (CAGCCAAAGTTGATGACTTTATGCAATTTACTG), T278A (GGTGGTAGGGTCATAGCAGTGAAATTTGGTGAC), T278D (GGTGGTAGGGTCATAGACGTGAAATTTGGTGAC). Site-directed mutagenesis was performed according to the manufacturer's instructions and the mutated sequences were verified. Mutant plasmids were finally transformed into BL21 *E. coli* strain for production of mutant proteins.

Purification of rat brain Ca^{2+}/calmodulin kinase II (CaMKII)

Native CaMKII (composed of autophosphorylatable subunits of M_r 50 000 and 60 000/58 000) was purified from rat forebrain (McGuiness *et al.*, 1985).

Determination of protein concentration

Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, USA) according to the manufacturer's instructions.

Protein electrophoresis

Gel electrophoresis under denaturing conditions was performed in the presence of SDS (Laemmli, 1970). The stacking and separating gels contained 4 and 15% (w/v) polyacrylamide respectively (acrylamide/bisacrylamide, 38:2). The gels were stained overnight with Coomassie brilliant blue R 250 (0.005% dye in 10% acetic acid, 10% isopropanol) and destained for 1.5 h in three changes of 10% acetic acid and 5% methanol.

Dephosphorylation and phosphorylation reactions

Dephosphorylation of proteins in pea nuclear extracts protein or purified recombinant hGT-1 was carried out with calf intestine phosphatase (CIP). Samples containing GT-1 activity (3 μg protein from nuclear extract or 1 μg hGT-1) in 25 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$ were incubated with 0.1 unit of CIP for 30 min at room temperature in the presence of various concentrations of NaF. The binding activity of the mixture to a BoxII tetramer (4BoxII) was then analyzed by DNA mobility shift assays. Phosphorylation of recombinant hGT-1 protein was performed using various kinases. For phosphorylation by casein kinase II (CKII) the reaction mixture contained 1 unit of CKII, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 1 mM ATP and 0.5 μCi γ-^{32}P-ATP. For phosphorylation by calcium/calmodulin-dependent kinase II (CaMKII) the reaction mixture contained various amounts of CaMKII, 25 mM HEPES pH 7.5, $Mg(CH_3COO)_2$, 0.1 mM EGTA, 0.05 mM DTT, 1.5 mM $CaCl_2$, 20 μg/ml calmodulin, 1 mM ATP and 0.5 μCi γ^{32}P-ATP. Phosphorylation by pea nuclear extracts was performed under both conditions described above. The reaction mixture was incubated for 30 min at 37 °C and stopped by precipitation with 10% (w/v) trichloracetic acid. After centrifugation at 14 000 × g for 5 min, the resulting protein pellet was washed twice with ice-cold acetone. The pellet was solubilized in SDS sample buffer (125 mM Tris-HCl pH 6.8, 360 mM 2-mercaptoethanol, 1.5% (w/v) SDS, 7.5% (w/v) glycerol, 0.5% (w/v) bromophenol blue). The polypeptides were separated by SDS-PAGE and the radioactivity incorporated into the hGT-1 protein was determined by autoradiography with Kodak X-Omat AR films and intensifying screens at −80 °C. When samples were assayed for DNA-binding activity in the presence of radiolabelled 4BoxII DNA probe, γ^{32}P-ATP was omitted from the hGT-1 phosphorylation reaction mixture. Various inhibitors were added as described in the text.

Phosphoamino acid analysis

Phosphoamino acid identification mapping was carried out as previously described (Boyle *et al.*, 1991). After treatment with various kinases and γ^{32}P-ATP, phosphorylated hGT-1 protein was immunoprecipitated, separated by SDS-PAGE, and transferred electrophoretically onto an Immobilon (Millipore, Bedford, MA) membrane. The membrane fragment containing the labelled protein was subjected to partial acid hydrolysis in the presence of 6 M HCl for subsequent phosphoamino-acid analysis. Radiolabelled phosphoamino acids were separated and identified by thin-layer electrophoresis at pH 3.5 in acetic acid/pyridine/H_2O (5:0.5:95, v/v) and autoradiography with Kodak X-Omat AR films and intensifying screens at −80 °C.

Mass spectrometry

Low-energy covalent bonds that originate from phosphorylation dissociate preferentially to produce characteristic mass spectrometric signatures that proved useful for the accurate identification and characterization of phosphorylated amino acids (Qin *et al.*, 1996). Analyses of amino acid-specific phosphorylation signatures were performed using a matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometer (Beavis and Chait, 1990) and a MALDI-ion trap mass spectrometer (Qin *et al.*, 1996), both constructed at Rockefeller University. The various hGT-1 samples were visualized on SDS-PAGE gels by copper staining (BioRad). The bands of interest were cut out, destained, and the protein subjected to in-gel digestion with trypsin or endoprotease Lys-C (0.5 μg enzyme in 100 mM Tris-HCl buffer, pH 8.3, for 2 h). The resulting proteolytic products were extracted from the gel in digestion buffer, followed by sequential extraction using 1:1 (v/v) of 1% aqueous TFA/acetonitrile and neat acetonitrile. These three extraction solutions were pooled, evaporated to dryness, and reconstituted in a small volume (5 μl) of 2:1 (v/v) of 0.1% aqueous TFA/acetonitrile. One μl of the peptide mixture was added to either 1 μl of 2,5-dihydroxybenzoic acid matrix (in 1:1 (v/v) water/acetonitrile) for MALDI-ion trap analysis or α-cyano-4-hydroxycinnamic acid matrix (5 g/l in 2:1 (v/v) of 0.1% aqueous TFA/acetonitrile) for MALDI-time-of-flight analysis. Mass spectra were obtained by adding individual spectra from 100–200 laser shots to improve the quality of the spectra.

Electrophoretic mobility shift studies

DNA-protein binding was performed in a solution (30 μl) containing 25 mM HEPES pH 7.6, 5% glycerol, 5 mM KCl, 1.1 mM EDTA, 1 mM DTT, 0.07 mg/ml poly(dI:dC) and 2000 cpm of radioactively labelled probe. This probe is a tetrameric oligonucleotide of *rbcS-3A* BoxII (4BoxII) and has been previously described (Hiratsuka *et al.*, 1994). After a 20-min incubation at 37 °C, the samples were separated on a 1% agarose gel in 10 mM Tris-HCl pH 7.4 and 1 mM EDTA. The gels were dried on DE81 ion exchange chromatography papers (Whatman) and autoradiographed. Band intensity was determined using a computing Phosphoimager from Molecular Dynamics.

Results

Alkaline phosphatase treatment inhibits GT-1 DNA-binding activity from light-grown pea nuclear extracts

We sought for physiological conditions in which GT-1 phosphorylation state could be involved in modulating its DNA binding *in vivo*. For this purpose, we used nuclear extracts from light-grown and etiolated pea seedlings. Freshly prepared nuclear extracts were incubated in the presence of calf intestine phosphatase (CIP) prior to assays of GT-1 DNA-binding activity. Figure 2 shows that BoxII DNA mobility shift was greatly inhibited when nuclear extracts from light-grown pea were treated with CIP (Figure 2, lane 1), as compared to untreated nuclear extracts (Figure 2, lane 2). A longer exposure of the autoradiogram showed that although the DNA binding was strongly inhibited by CIP treatment, it was not completely abolished and the remaining GT-1/BoxII complex was still detectable. Addition of NaF into the incubation medium protected the DNA-binding activity from being affected by the CIP treatment (Figure 2, lanes 3–5). Because NaF is an inhibitor of CIP activity, this result showed that BoxII binding activity in nuclear extracts from light-grown pea likely involves a phosphorylated factor, presumably GT-1, which was inactivated by dephosphorylation. By contrast, etiolated pea nuclear extracts exhibit a GT-1 DNA-binding activity which was not affected by CIP treatment (Figure 2, lanes 8–9). This latter result shows that, in etiolated pea, GT-1 binding activity is weaker than in light-grown pea, and is independent of phosphorylation.

Purification of recombinant hGT-1

To address whether GT-1 phosphorylation state could influence GT-1 DNA-binding activity, we used a full-length cDNA clone encoding GT-1 from *Arabidopsis* (Hiratsuka *et al.*, 1994) to produce recombinant protein in *E. coli*. The cDNA was inserted into the expression vector pET-15b (see Materials and methods) containing six histidine codons immediately upstream of the initiation codon. The resulting fusion gene encoded a GT-1 protein with an extra 6 histidines at the N-terminus, and this was referred to as 'hGT-1'. Recombinant hGT-1 can be rapidly purified in a single-step procedure using immobilized metal affinity chromatography. Figure 3 shows that after induction with isopropylthiogalactoside (IPTG), hGT-1 comprised ca. 1–2% of total *E. coli* cellular

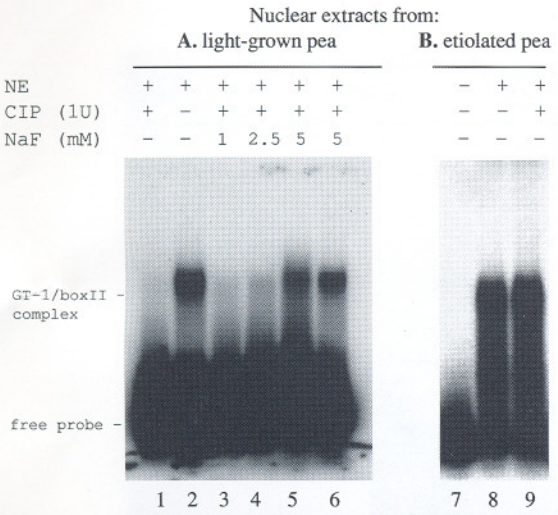

Figure 2. Calf intestine phosphatase treatment inhibits GT-1 binding activity of nuclear extracts from light-grown pea plants. Dephosphorylation was performed with calf intestine alkaline phosphatase (CIP). A. Nuclear extracts from light-grown pea seedlings. 1 μg protein of nuclear extracts was incubated in 10 μl of CIP buffer for 30 min at room temperature under the following conditions: lane 2, nuclear extract only; lane 1, 1 unit CIP; lane 3, 1 unit CIP and 1 mM NaF; lane 4, 1 unit CIP and 2.5 mM NaF; lane 5, 1 unit CIP and 5 mM NaF; lane 6, 5 mM NaF. B. Nuclear extracts from etiolated pea seedlings. Nuclear extracts (2 μg protein) from etiolated pea were treated in 10 μl of CIP buffer for 30 min at room temperature under the following conditions: lane 7, no nuclear extract; lane 8, 2 μg nuclear extract; lane 9, 2 μg nuclear extract and 1 unit CIP. After incubation, the volume was adjusted to 30 μl with the gel retardation buffer containing the radiolabelled probe and assayed as described in Materials and methods.

protein. The predicted molecular mass of the protein was 48.762 kDa although it migrated in SDS-PAGE gels with an apparent mass of 51 kDa. Ca. 90% of hGT-1 in *E. coli* was found in inclusion bodies. In the soluble protein fraction, the N-terminal cluster of histidines appeared to be exposed to the surface of the protein, so that hGT-1 protein was retained on the Ni^{2+}-affinity column (Figure 3, lane 3). Purified hGT-1 fraction also contained small amounts of low-molecular-weight polypeptides (Figure 3, lane 3) which we identified, by cross-reactivity with anti-GT-1 polyclonal mouse antibodies and mass spectrometry analysis, as being degradation products of the full-length protein (data not shown).

In confirmation of previous results (Hiratsuka *et al.*, 1994), we found that the DNA-binding properties of hGT-1 were indistinguishable from those of native GT-1 from pea nuclear extracts as judged by DNA competition assays and differential binding to critical mutants of BoxII (data not shown).

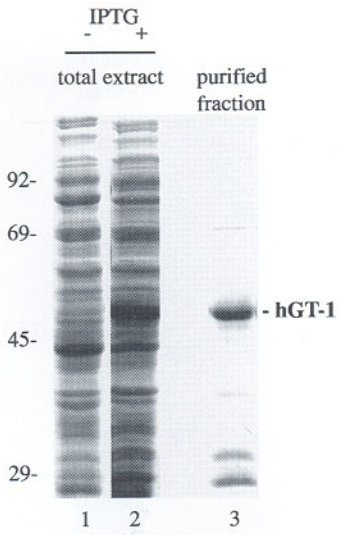

Figure 3. Expression and purification and of hGT-1. hGT-1 fractions were subjected to SDS-PAGE followed by staining with Coomassie blue. Lane 1, total protein of non-induced *E. coli* BL21 cells (30 μg); lane 2, total protein of *E. coli* BL21 cells (30 μg) after a 3-h induction with IPTG, 0.4 mM; lane 3, native hGT-1 (2 μg) purified by nickel affinity chromatography. Molecular mass markers in kDa are given on the left.

Recombinant hGT-1 is a putative substrate for various kinase sources

We investigated whether hGT-1 could be a substrate for various kinases. We used nuclear extracts from pea leaves as a source for a putative plant kinase activity presumably responsible for phosphorylating GT-1 *in vivo*. Pea was selected for these studies because nuclei from light-grown and etiolated seedlings, and large amounts of material for biochemical studies can be obtained. Nuclear extracts from light-grown pea seedlings catalyzed the transfer of phosphate from γ^{32}P-ATP to hGT-1 (Figure 4A).

Phosphorylation of hGT-1 by purified mammalian serine/threonine kinases was also carried out using casein kinase II (CKII) and multifunctional calcium/calmodulin-dependent protein kinase II (CaMKII) (see Materials and methods). Both CKII and CaMKII phosphorylated hGT-1 *in vitro* (Figure 4B and 4C) as expected from the presence of the respective consensus substrate motifs in hGT-1 sequence (Figure 1). Autophosphorylation of α and β subunits of CaMKII (migrating respectively around 50 and 60 kDa) was detected (Figure 4B, lane 6). In the absence of calcium and calmodulin, a weak phosphorylation of added hGT-1 was detected (Figure 4B, lane 2), which was considerably enhanced in

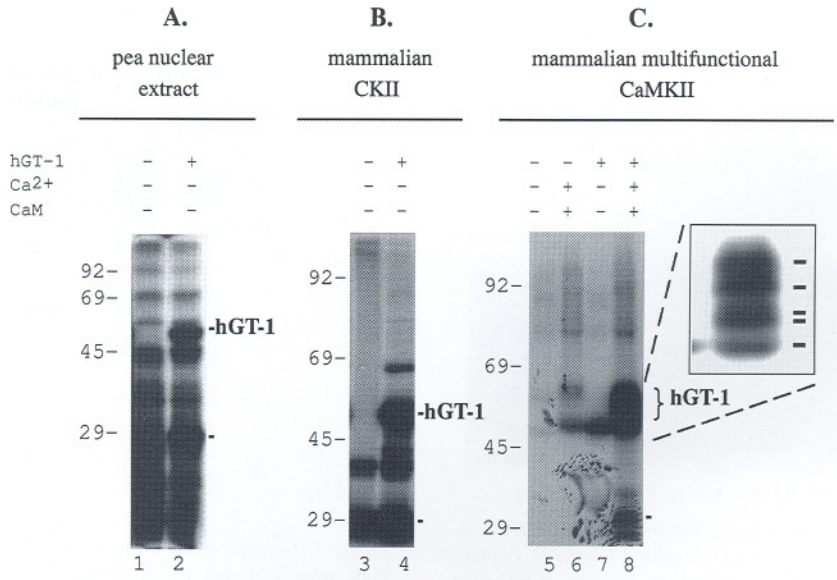

Figure 4. In vitro phosphorylation of hGT-1 by various kinases. Phosphorylated proteins were separated by SDS-PAGE and radioactivity incorporated into hGT-1 was determined by autoradiography. A. Phosphorylation of GT-1 by nuclear extracts from light-grown pea (in CaMKII buffer) without addition of calcium and calmodulin. Lane 1, 2 μg of nuclear extract (NE); lane 2, 2 μg of NE with 1 μg of hGT-1 purified by nickel affinity chromatography. B. Phosphorylation by CKII. Lane 3, CKII (20 ng) alone; lane 4, CKII with 1 μg of native hGT-1. C. Phosphorylation by CaMKII. Lane 5, CaMKII (20 ng) alone; lane 6, CaMKII (20 ng) with $CaCl_2$ (1.5 mM) and calmodulin (20 μg/ml); lane 7, CaMKII (20 ng) with 1 μg of hGT-1; lane 8, CaMKII (20 ng) with $CaCl_2$ (1.5 mM), calmodulin (20 μg/ml) and 1 μg of hGT-1. No marked phosphorylation was noted when CaMKII was incubated with $CaCl_2$ or calmodulin alone (data not shown). Portions of the autoradiogram in lane 8 were enlarged as an insert to show the multiple forms of phosphorylated hGT-1. Molecular mass markers in kDa are given on the left.

the presence of both calcium and calmodulin (Figure 4B, lane 8). After phosphorylation by CaMKII, radiolabelled hGT-1 migrated as a broad band composed mostly of 4 major bands (Figure 4C, magnified insert).

Serine and threonine residues from hGT-1 are phosphorylated by various kinases

In all cases, analyses of labelled amino acids showed that the phosphorylated residues were serine and threonine (Figure 5). Because hGT-1 was strongly phosphorylated by treatment with CaMKII (Figure 4C, magnified insert), we could analyze peptides generated by trypsin or LysC digestion of phosphorylated hGT-1 using matrix-assisted laser desorption/ionization mass spectrometry (see Materials and methods). Amongst the six potential sites for CaMKII modification (Figure 1), T133 and S198 were found to be phosphorylated, whereas no phosphorylation was detected on T86, S175 or T278 (data not shown). The phosphorylation status of T179 was not determined. These results suggest that, although the multifunctional CaMKII has a broad specificity for its substrates (i.e. R-X-X-S/T and K-X-X-S/T motifs), all potential sites are not equally phosphorylated.

Phosphorylation of hGT-1 influences DNA-binding activity in vitro

We investigated the effect of hGT-1 phosphorylation *in vitro* on DNA binding. We found that treatment of hGT-1 with calf intestine phosphatase (CIP) had little effect on hGT-1 binding to BoxII (Figure 6A, lanes 2, 3). This result supports the assumption that hGT-1 purified from bacteria was either not phosphorylated, or only poorly phosphorylated. It should be noted that untreated hGT-1 or hGT-1 treated with CIP showed significant binding activity. To examine the effects of phosphorylation on hGT-1 activity, we treated hGT-1 with various purified kinases *in vitro* and assayed the modified hGT-1 by gel shift assays (Figure 6, B to D). Treatments with mammalian CKII had no effect on GT-1 binding to BoxII (Figure 6D). By contrast, incubation of hGT-1 in presence of ATP and CaMKII (activated by Ca^{2+} and calmodulin) stimulated DNA-binding activity to BoxII (Figure 6B, lanes 5, 6). A 10- to 20-fold increase in DNA binding could be routinely measured by electrophoretic DNA mobil-

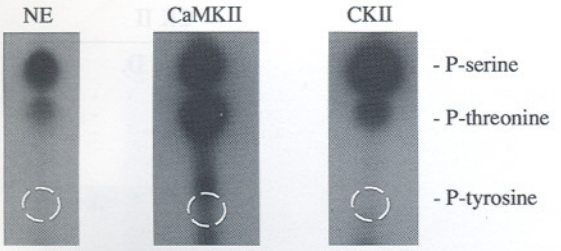

Figure 5. Identification of phosphorylated amino acids in hGT-1 modified by various kinases. Hydrolysis products of phosphorylated hGT-1 were separated by thin-layer electrophoresis at pH 3.5 and subjected to autoradiography. P-Serine, phosphoserine; P-Threonine, phosphothreonine; P-Tyrosine, phosphotyrosine. Phosphorylation was performed in the presence of nuclear extracts (NE) prepared from light-grown pea plants or calcium/calmodulin kinase II from rat brain (CaMKII) or mammalian casein kinase II (CKII).

ity shift. Addition of a specific inhibitor of CaMKII, KN93, inhibited the activation by CaMKII, whereas a non-active analogue, KN92, did not block the activation (Figure 6C, lanes 12 and 13 respectively).

Our results show that hGT-1 DNA-binding activity can be modulated by phosphorylation. Because the marked phosphorylation of hGT-1 by CKII had no effect on DNA binding, whereas CaMKII phosphorylation increased DNA-binding activity of hGT-1, key serine and/or threonine residues phosphorylated by CaMKII are likely to involved in hGT-1/BoxII interaction.

GT-1 site-directed mutagenesis on CaMKII phosphorylatable residues affects DNA-binding activity in vitro

To identify critical serine and/or threonine residues involved in DNA binding, we constructed point mutations of hGT-1 on the six amino acids in CaMKII substrate consensus domains (Figure 1). Serine or threonine was replaced by the neutral non-phosphorylatable amino acid alanine (T86A, T133A, S175A, T179A, S198A and T278A), or the negatively charged amino acid aspartic acid, which can mimic a permanently phosphorylated residue (T86D, T133D, S175D, T179D, S198D and T278D). The 12 mutant cDNAs were expressed in the inducible *E. coli* strain BL21, and the recombinant proteins purified by Ni^{2+}-chelate chromatography. The quality of the purified mutant proteins was monitored by SDS-PAGE and was found indistinguishable from that of the WT hGT-1 preparation (data not shown). Table 1 shows that the BoxII-binding activities of wild type (WT), and the T86A, T86D, S175A, S175D, T179A, T179D, T278A and T278D mutants were similar *in vitro*. Significant differences, however, were detected in mutations affecting Thr-133 and Ser-198 (Figure 7 and Table 1). Mutation of T133 to D133 led to an increase in DNA-binding activity whereas a slight decrease in BoxII binding was found when T133 was changed to A133 (Figure 7A). By contrast, mutation of S198 to A198 slightly increased DNA-binding activity, whereas the presence of an aspartic acid in position 198 decreased the binding (Figure 7B).

Because opposing effects were obtained when T133 and S198 were changed to aspartic acid residues, we also analyzed the DNA-binding activity of the double-mutant (T133D/S198D). Interestingly, the DNA-binding activity of the double mutant, T133D/S198D, was similar to that of the single T133D mutant (Figure 7C). This result suggests that phosphorylation on both T133 and S198 (as mediated by mammalian CaMKII) activates DNA binding to a comparable degree as phosphorylation of T133 alone.

Table 1. Mutations of serine and threonine residues of hGT-1 modify its DNA-binding to *rbcS-3A* BoxII *in vitro*. DNA binding was quantified in DNA-shift experiments using 1 μg of purified WT or mutant hGT-1. DNA binding is given as percentage of WT DNA binding. Error is given for $n = 2$, except ([a]): $n = 5$ and ([b]): $n = 6$. The purity of WT and mutant hGT-1 proteins was verified by SDS-PAGE and found to be more than 90%. We assume that the proportions of active hGT-1 proteins (WT and mutants) in the protein purification are similar, and that differences in binding activities are mostly due to differences in specific binding activity (see text).

hGT-1 point mutation	% of BoxII DNA binding by hGT-1 (WT)
hGT-1(WT)	100
hGT-1(T86A)	87.5 (± 2.5)
hGT-1(T86D)	125 (± 5)
hGT-1(T133A)	53 (± 24)[a]
hGT-1(T133D)	265 (± 134)[a]
hGT-1(S175A)	110 (± 10)
hGT-1(S175D)	87.5 (± 12.5)
hGT-1(T179A)	125 (± 15)
hGT-1(T179D)	120 (± 20)
hGT-1(S198A)	119 (± 7)[b]
hGT-1(S198D)	12.5 (± 10)[b]
hGT-1(T278A)	80 (± 0)
hGT-1(T278D)	95 (± 5)

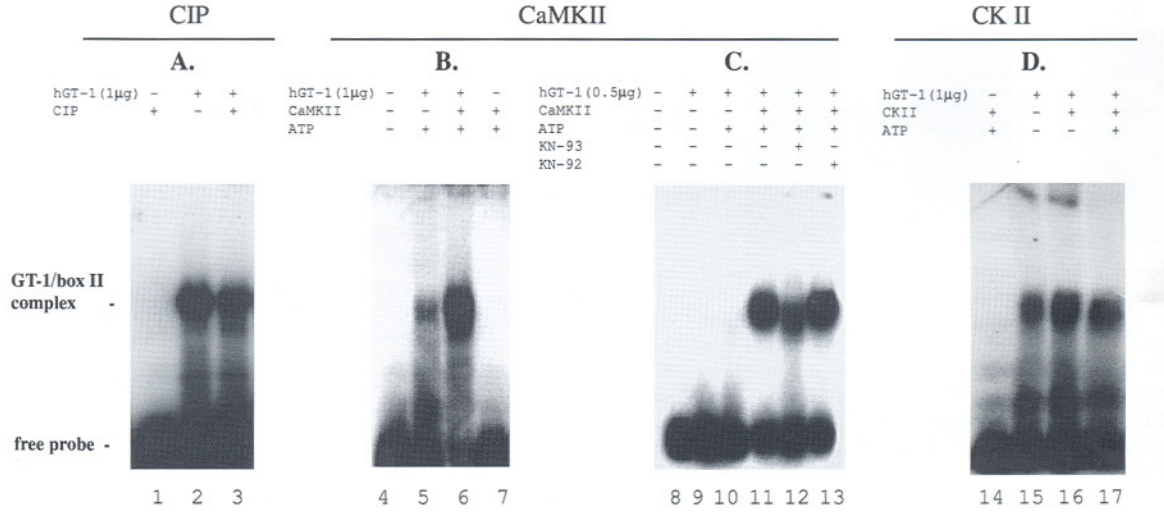

Figure 6. Binding of hGT-1 to *rbcS-3A* BoxII is modulated hy phosphorylation. The following components were preincubated for 30 min at 37 °C in 10 μl of CIP buffer (A), or CaMKII buffer containing calcium and calmodulin (B and C), or CKII buffer (D). Amounts of hGT-1 were adjusted to 0.5 or 1 μg (as indicated), and exposure times vary between panels. A. Treatment with CIP. Lane 1, free DNA probe alone; lane 2, hGT-1 (1 μg); lane 3, hGT-1 and CIP (1 unit). B. and C. Treatment with CaMKII. Lanes 4 and 8, free DNA probe alone; lane 5, hGT-1 (1 μg) and ATP (1 mM); lane 6, hGT-1 (1 μg), CaMKII (20 ng) and ATP (1 mM); lane 7, CaMKII (20 ng) and 1 mM ATP; lane 9, hGT-1 (0.5 μg); lane 10, hGT-1 (0.5 μg) and CaMKII (20 ng); lane 11, hGT-1 (0.5 μg), CaMKII (20 ng) and ATP (1 mM); lane 12, hGT-1 (0.5 μg), CaMKII (20 ng), KN-93 (10 μM) and ATP (1 mM); lane 13, hGT-1 (0.5 μg), CaMKII (20 ng), KN-92 (10 μM) and ATP (1 mM). C. Treatment with CKII. Lane 14, CKII (20 ng) and ATP (1 mM); lane 15, hGT-1 (1 μg); lane 16, hGT-1 (1 μg) and CKII (20 ng); lane 17, hGT-1 (1 μg), CKII (20 ng) and ATP (1 mM). After each incubation, the volume in each tube was adjusted to 30 μl with gel retardation buffer containing the radiolabelled probe, and assayed as described in Materials and methods. Note that different exposure times were used for the different panels.

Discussion

The regulation of *trans*-acting factors, such as GT-1, by phosphorylation and dephosphorylation is likely a very complex process. For example, phosphorylations/dephosphorylations could be the key post-translational modifications for GT-1 stability, the migration of GT-1 from the cytosol to the nucleus, the oligomerization of the factor itself, the interaction of GT-1 with target DNA sequences and the interactions of GT-1 with proteins of the transcriptional machinery and other *trans*-acting factors. As a first step to understanding these complex processes, we used a biochemical approach and focused our investigations on the effects of dephosphorylation and phosphorylation on the DNA-binding activity of GT-1 *in vitro*.

Constitutive binding of non-phosphorylated GT-1

Although GT-1 specifically binds to BoxII, which was demonstrated to be critical for control of gene expression by light, no major difference has yet been found between GT-1 activities from nuclear extracts from light-grown and etiolated plants. Green *et al.* (1987, 1988) have reported that nuclear extracts from both light-grown and dark-adapted plants contain a DNA-binding activity with the characteristics of GT-1, with respect to BoxII binding site sequence specificity. Here, we showed that nuclear extracts of etiolated pea plants contained a GT-1 activity as well. It is difficult to quantitatively compare the specific activities of GT-1 between nuclear extracts of etiolated and light-grown seedlings because of variable recovery of GT-1 DNA-binding activity from preparation to preparation. Moreover, extracts from light-grown plants are likely to contain considerably more Rubisco contamination than those of etiolated plants. Notwithstanding these problems, we detected a qualitative difference between nuclear extracts derived from the two sources: GT-1 activity of nuclear extracts from light-grown pea seedlings was sensitive to a phosphatase treatment, whereas the GT-1 activity of nuclear extracts from etiolated pea seedlings was not. These results led us to assume that in the dark there is a basal level of constitutive GT-1 binding to its target sites, which is presumably increased by light through phosphorylation. Accordingly, we focused on the effects of phosphorylation on this constitutive GT-1 binding activity.

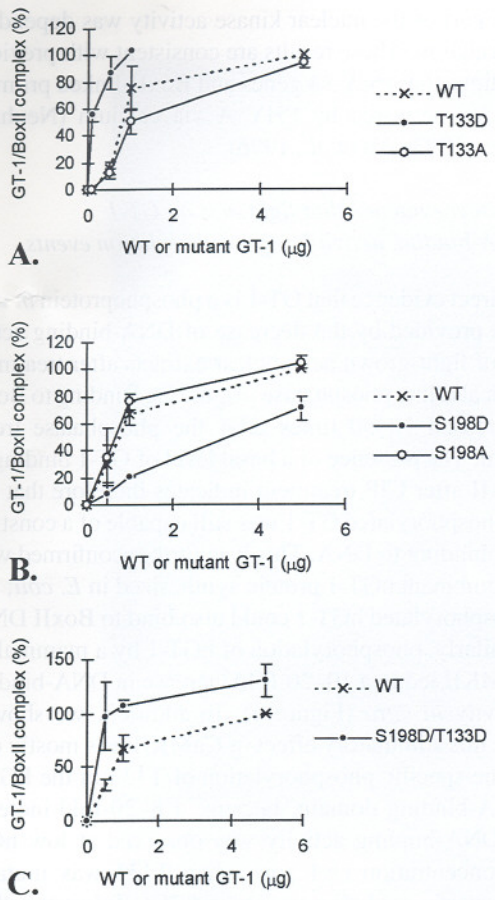

Figure 7. Quantitative DNA-binding analysis of hGT-1 mutated in T133 and S198. DNA-binding was performed at a constant probe concentration and increasing protein concentrations. The amount of bound complex was measured by a phosphorimager. Relative phosphorimager readings were normalized by comparison to the binding of 5 μg of WT hGT-1 run on the same gel. Mobility shift assays with hGT-1 mutants. A. T133A and T133D. B. S198A and S198D. C. T133D/S198D. Data presented in A., B. and C. are the means of 5, 3 and 2 independent assays respectively.

Recombinant hGT-1 displayed a DNA-binding activity similar to that obtained from nuclear extracts of both light-grown and etiolated pea nuclear extracts. Its specific binding activity was low: we estimated that 0.5–1 μg of purified hGT-1 were necessary to obtain equivalent DNA-binding activity from 1 μg of light-grown and 2 μg of etiolated pea nuclear extracts. The low specific activity of purified hGT-1 may be due to the low constitutive binding activity of the unphosphorylated hGT-1; it is also possible that only a part of the recombinant proteins was active. We found that recombinant hGT-1 binds constitutively to BoxII, and the binding activity was either slightly or not affected at all by phosphatase treatment (Figure 6A). On the other hand, GT-1 DNA-binding activity of nuclear extracts from light-grown seedlings was reduced 10–50-fold upon phosphatase treatment. Taking these results into consideration, it is possible that GT-1 binding to its target site *in vivo* is modulated by residue-specific phosphorylation and dephosphorylation.

Casein kinase II (CKII) is likely not involved in the regulation of GT-1 DNA-binding activity

In previous studies, DNA binding activities of other factors that can potentially interact with the pea *rbcS-3A* promoter were shown to be regulated by phosphorylation through a plant nuclear CKII activity. Datta and Cashmore (1989) reported that a factor, AT-1, binds to a specific AT-rich element (the AT-1 box) within the promoter sequence of *rbcS-3A*. The DNA-binding activity of AT-1 was abolished by phosphorylation by a kinase that used both Mg-ATP and Mg-GTP as substrates, and was inhibited by spermine. These properties are consistent with those displayed by CKII. Likewise, Klimczak *et al.* (1992) showed that recombinant *Arabidopsis* GBF-1 was a substrate for a plant nuclear CKII activity. Phosphorylation of GBF-1 by plant CKII stimulated its binding to the G-box, a DNA sequence present within the *rbcS-3A* promoter. Because GT-1 can also be phosphorylated by a CKII-like activity from nuclear extracts, we investigated the effects of this phosphorylation on GT-1 DNA-binding activity. We found no significant difference in the DNA-binding activity of hGT-1 and hGT-1 treated with CKII (Figure 6), thus ruling out a regulation by this kinase on *in vivo* binding of GT-1 to its target DNA. However, we cannot exclude that CKII may modulate other aspects of GT-1 activity *in vivo*, such as the translocation of GT-1 into the nucleus and its interaction with other transcription factors.

T133 and S198 are important for binding of hGT-1 to rbcS-3A *BoxII*

Serine and threonine residues in the mammalian CaMKII and plant CDPKs common consensus motifs (basic-X-X-serine/threonine) are localized in the vicinity of the putative trihelix DNA-binding region of GT-1. T86 is one of the first amino acids in the putative α-helix 1/α-helix 2 domain, T133 is between α-helix 2 and α-helix 3, and S175 and T179 are at the C-terminus of α-helix 4 (Figure 1). These residues are therefore potential sites for regulating GT-1 DNA-binding activity. In addition, rearrangement

of α-helices by phosphorylation might lead to conformational changes in GT-1 structure, thus affecting its interaction with other components of the transcription machinery.

Indeed, we found that phosphorylation of hGT-1 with mammalian CaMKII led to a marked stimulation of DNA binding to *rbcS-3A* BoxII *in vitro*. Phosphorylation of hGT-1 by CaMKII was strong (Figure 4) indicating that more than one of the putative phosphorylation sites for CaMKII in hGT-1 were modified. Mass spectrometry analyses showed that all the 6 putative sites were not identically phosphorylated by CaMKII; phosphorylation was best achieved on T133 and S198. T133 and S198 residues are therefore likely exposed on the surface of the protein in a favorable conformation for CaMKII phosphorylation.

Mutational analyses showed that amongst all the serine and threonine residues in basic-X-X-S/T domains, T133 and S198 were the most important regulators of hGT-1 DNA binding. Surprisingly, when these residues were mutated to aspartic acids, opposing effects were observed. The T133D mutant bound BoxII better than WT hGT-1, whereas the opposite result was obtained with the S198D mutant. In the case of the double-mutant T133D/S198D, the overall effect on hGT-1 DNA-binding was similar to the stimulatory effect of the T133D mutation alone (see Figure 7). This result suggests that, with respect to hGT-1 binding activity, the T133D mutation was dominant over the S198D mutation.

To our knowledge, only one homologue of the multifunctional CaMKII from yeast and animal cells has been reported in plants (Watillon *et al.*, 1993), but this homology has not yet been verified at the functional level by biochemical experiments. The report of an anther-specific CaMK with a visilin-like domain (Patil *et al.*, 1995) supports the existence of more specialized CaMKs in plants involved in Ca^{2+}/CaM-mediated phosphorylations. Calcium-dependent protein kinases (CDPKs) are a ubiquitous family of plant protein kinases sharing limited enzymatic similarities with CaMKII. They phosphorylate proteins mostly on basic-X-X-S (Weaver and Roberts, 1992). Unlike CaMKII, CDPKs do not require calmodulin, and are more selective about substrate structural feactures in the vicinity of the phosphorylation site (Weaver and Roberts, 1992). CDPKs might be involved in the activation of genes under the control of BoxII by phosphorylating GT-1. We noted that the phosphorylation of hGT-1 *in vitro* by pea nuclear extracts was partially inhibited by 1 mM EGTA (data not shown), suggesting that part of the nuclear kinase activity was dependent on calcium. These results are consistent with previous findings that *rbcS-3A* genes and BoxII-linked promoters are activated by PHY A via calcium (Neuhaus *et al.*, 1993; Wu *et al.*, 1996).

Indirect evidence that light acts on GT-1 DNA-binding activity by phosphorylation events

Indirect evidence that GT-1 is a phosphoprotein *in vivo* was provided by the decrease of DNA-binding activity of light-grown pea nuclear extracts after treatment with alkaline phosphatase (Figure 2). Binding to BoxII decreased 10–50 times after the phosphatase treatment. The presence of a basal level of GT-1 binding to BoxII after CIP treatment indicates therefore that the dephosphorylated GT-1 was still capable of a constitutive binding to DNA. This was further confirmed with recombinant hGT-1 protein synthesized in *E. coli*; unphosphorylated hGT-1 could also bind to BoxII DNA. Similarly, phosphorylation of hGT-1 by a mammalian CaMKII led to a 10–20-fold increase in DNA-binding activity *in vitro* (Figure 6). In addition, we showed that this stimulatory effect of CaMKII was mostly due to the specific phosphorylation of T133 in the hGT-1 DNA-binding domain, because a 8–20-fold increase of DNA-binding activity was observed at low hGT-1 concentration (<1 μg) when T133 was mutated to aspartic acid (Figure 7A and 7C). Taken together, these results lead us to propose a working hypothesis for future investigations: GT-1 could act as a non-productive binding factor to BoxII in the dark, but be converted into an activator in the light, whose binding activity for BoxII increases after phosphorylation of specific residues (such as T133) in the DNA-binding domain. Fine modulation of this molecular switch could be further brought about by combinations of phosphorylations and dephosphorylations of various GT-1 residues (phosphorylation of some residues such as S198 could, for example, participate in decreasing the overall activity of GT-1).

The modulation of GT-1 by combination of phosphorylations of various residues may be a key mechanism by witch GT-1 could bind to other sites, such as the GATA motif, and be involved in the fine tuning of genes in regulation by circadian rhythm (Teakle and Kay, 1995). Identifying endogenous phosphatase(s) and kinase(s) that modulate GT-1 activity is clearly a future challenge, and further studies such as analyses of transgenic plants containing GT-1 mutant trans-

genes may eventually lead to the characterization of GT-1 function and modulators *in vivo*.

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References

Beavis, R.C. and Chait, B. 1990. Rapid, sensitive analysis of protein mixtures by mass spectrometry. Proc. Natl. Acad. Sci. USA 87: 6873–6877.

Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N.H. 1994. Cyclic GMP and calcium mediate phytochrome phototransduction. Cell 77: 73–81.

Boyle, W.J., van der Geer, P. and Hunter, T. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Meth. Enzymol. 201: 110–149.

Chattopadhyay, S., Puente, P., Deng, X.W. and Wei, N. 1998. Combinatorial interaction of light-responsive elements plays a critical role in determining the response characteristics of light-regulated promoters in *Arabidopsis*. Plant J. 15: 69–77.

Datta, N. and Cashmore, A.R. 1989. Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell 1: 1069–1077.

Dehesh, K., Bruce, W.B. and Quail, P.H. 1990. A *trans*-acting factor that binds to a GT-motif in a phytochrome gene promoter. Science 250: 1397–1399.

Furuya, M. 1993. Phytochromes: their molecular species, gene families, and functions. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 617–645.

Gallagher, T.F. and Ellis, R.J. 1982. Light-stimulated transcription of genes for two chloroplast polypeptides in isolated leaf nuclei. EMBO J. 1: 1493–1498.

Gilmartin, P.M., Sarokin, I., Memelink, J. and Chua, N.H. 1990. Molecular switches for plant genes. Plant Cell 2: 369–378.

Gilmartin, P.M., Memelink, J., Hiratsuka, K., Kay, S.A. and Chua, N.H. 1992. Characterization of a gene encoding a DNA-binding protein with specificity for a light-responsive element. Plant Cell 4: 839–849.

Green, P.J., Kay, S.A. and Chua, N.H. 1987. Sequence-specific interaction of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. EMBO J. 6: 2543–2549.

Green, P.J., Yong, M.H., Cuozo, M., Kano-Murakami, Y., Silverstein, P. and Chua, N.H. 1988. Binding site requirements for pea nuclear protein factor GT-1 correlate with sequence required for light-dependent transcriptional activation of the *rbcS-3A* gene. EMBO J. 7: 4035–4044.

Harter, K., Kircher, S., Frohnmeyer, H., Nagy, F. and Schäfer, E. 1994. Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. Plant Cell 6: 545–559.

Hiratsuka, K., Wu, X., Fukuzawa, H. and Chua, N.H. 1994. Molecular dissection of GT-1 from *Arabidopsis*. Plant Cell 6: 1805–1813.

Kim, B.C., Tenessen, D.J. and Last, R.L. 1998. UV-B-induced photomorphogenesis in *Arabidopsis thaliana*. Plant J. 5: 667–674.

Klimczak, L.J., Schindler, U. and Cashmore, A.R. 1992. DNA-binding activity of the *Arabidopsis* G-box binding factor GBF1 is stimulated by phosphorylation by casein kinase II from broccoli. Plant Cell 4: 87–98.

Kuhlemeier, C., Fluhr, R., Green, P.J. and Chua, N.H. 1987. Sequences in the pea *rbcS-3A* gene have homology to constitutive mammalian enhancers but function as negative regulatory elements. Genes Dev. 1: 247–225.

Kuhlemeier, C., Cuozzo, M., Green, P.J., Goyvert, E., Ward, K. and Chua, N.H. 1988. Localization and conditional redundancy of regulatory elements in *rbcS-3A*, a pea gene encoding the small subunit of ribulose-bisphosphate carboxylase. Proc. Natl. Acad. Sci. USA 85: 4662–4666.

Kuhn, R.M., Caspar, T., Dehesh, K. and Quail, P.H. 1993. DNA-binding factor GT-2 from *Arabidopsis*. Plant Mol. Biol. 23: 337–348.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lam, E. 1995. Domain analysis of the plant DNA-binding protein GT1a: requirement of four putative α-helices for DNA-binding and identification of a novel oligomerization region. Mol. Cell. Biol. 15: 1014–1020.

Lam, E. and Chua, N.H. 1990. GT-1 binding site confers light responsive expression in transgenic tobacco. Science 248: 471–474.

Lawton, M.A., Dean, S.M., Dron, M., Kooter, J.M., Kragh, K.M., Harrisson, M.J., Yu, L., Tanguay, L., Dixon, R.A. and Lamb, C. 1991. Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1. Plant Mol. Biol.16: 235–249.

Lin, C., Ahmad, M. and Cashmore, A.R. 1996. *Arabidopsis* cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. Plant J. 5: 893–902.

McGuiness, T.L., Lai, Y. and Greengard, P. 1985. Ca^{2+}/calmodulin-dependent protein kinase II: isozymic forms from rat forebrain and cerebellum. J. Biol. Chem. 260: 1696–1704.

Neuhaus, C., Bowler, C., Kern, R. and Chua, N.H. 1993. Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell 73: 937–952.

Orozco, B.M. and Ogren, W.L. 1993. Localization of light-inducible and tissue-specific regions of the spinach ribulose bisphosphate carboxylase/oxygenase (rubisco) activase promoter in transgenic tobacco plants. Plant Mol. Biol. 23: 1129–1138.

Patil, S., Takezawa, D. and Poovaiah, B.W. 1995. Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visilin-like calcium-binding domain. Proc. Natl. Acad. Sci. USA 92: 4797–4801.

Pearson, R.B. and Kemp, B.E. 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Meth. Enzymol. 200: 62–81.

Perisic, O. and Lam, E. 1992. A tobacco DNA-binding protein that interacts with light-responsive box II element. Plant Cell 4: 831–838.

Puente, P., Wei, N. and Deng, X.W. 1996. Combinatorial interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene elements in *Arabidopsis*. EMBO J. 15: 3732–3743.

Qin, J., Ruud, J. and Chait, B. 1996. A practical ion trap mass spectrometer for the analysis of peptides by matrix-assisted laser desorption/ionisation. Anal. Chem. 68: 1784–1791.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Teakle, G.R. and Kay, S.A. 1995. The GATA-binding protein CGF-1 is closely related to GT-1. Plant Mol. Biol. 29: 1253–1266 (1995).

Watillon, B.R., Kettmann, R., Boxus, P. and Burny, A. 1993. A calcium/calmodulin-binding serine/threonine protein kinase homologous to the mammalian type II calcium/calmodulin-dependent protein kinase is expressed in plant cells. Plant Physiol. 101: 1381–1384.

Weaver, C.D. and Roberts, D.M. 1992. Determination of the site of phosphorylation of nodulin 26 by the calcium-dependent protein kinase from soybean nodules. Biochemistry 31: 8954–8959.

Wu, Y., Hiratsuka, K., Neuhaus, G. and Chua, N.H. 1996. Calcium and cGMP target distinct phytochrome-responsive elements. Plant J. 10: 1149–1154.