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Fine structure and function of the osmotin gene promoter

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Abstract

The gene encoding osmotin, a tobacco pathogenesis-related protein, has been shown to be regulated by an array of hormonal and environmental signals. The osmotin promoter fragment −248 to −108 upstream of the transcription start site (fragment A), was sufficient to direct reporter gene expression when fused to a minimal CaMV 35S promoter in transient assays using microprojectile bombardment. This was consistent with previous 5′-deletion analyses of the osmotin promoter which showed that the promoter sequence from −248 to −108 is absolutely required for reporter gene activity. Nuclear protein factors from salt-adapted tobacco cells, ABA-treated unadapted cells, and young cultured tobacco leaves were shown to interact with fragment A by gel mobility-shift assays. DNase I footprinting revealed that three conserved promoter elements in fragment A interact specifically with nuclear factors. These elements are: (1) a cluster of G-box-like sequences (G sequence); (2) an AT-1 box-like sequence, 5′-AATTATTT-TATG-3′ (AT sequence); (3) a sequence highly conserved in ethylene-induced PR gene promoters, 5′-TAAGA/CGCCGCC-3′ (PR sequence). Transient expression assays performed with fragment A deletions fused to GUS indicated that osmotin promoter activity correlated with the presence of these elements. UV cross-linking analysis showed that the protein complex bound to fragment A consisted of at least four individual proteins with approximate molecular masses of 28, 29, 40 and 42 kDa. One component of this protein complex, which was associated with the G sequence, was a 14-3-3 like protein.

Introduction

Osmotin is a basic 24 kDa protein which was first identified as the most prominent polypeptide in salt-adapted tobacco cells [46, 47]. Osmotin-like proteins have also been found in many other plant species, such as tomato [23, 58], potato [37], *Arabidopsis* [51], rice [41], and maize [54]. Osmotin is a member of Group 5 tobacco pathogenesis-related (PR) proteins [49, 4]. It has been shown recently that tobacco PR-proteins of Groups 1 to 5 exhibit antifungal activity and therefore could all have important plant defense functions [1, 7, 31, 39, 44].

The gene encoding osmotin has been cloned [48]. Regulation of its expression has been studied in wild-type tobacco plants and transgenic tobacco plants that carry the osmotin promoter

(−1595 to +45) fused to the β-glucuronidase reporter gene. These, and other studies have shown that expression of the osmotin gene can be induced by several hormonal and environmental signals such as salinity, drought, ABA, wounding, ethylene, salicylic acid, tobacco mosaic virus, fungal infection, UV light, cold and auxin [5, 25, 29, 31, 34, 59]. Regulation of gene expression by pathogens and associated signal molecules such as ethylene, salicylic acid and ABA is a property that osmotin shares with other PR proteins [15, 28, 56, 59].

Analysis of chimeric constructs containing 5′-deletion fragments of the osmotin promoter fused to a GUS reporter gene in a transient expression system [40] and in transgenic plants [25] indicated that sequences up to −248 upstream of the transcription start site are absolutely required for reporter gene expression. Reporter gene expression increased with promoter length and was maximum in a −1052 promoter fragment. The −248 to +45 promoter fragment::GUS fusion has been found to respond to the same hormonal and environmental signals tested so far as the full-length −1595 to +45 promoter [20, 24, 25, 34, 40, 59; P.-F.L. Chang and Y. Xu, unpublished data]. It was also sufficient for synergism between inducers [59] and exhibited the same spatial, temporal and developmental expression pattern as the full-length −1595 to +45 promoter [24]. Reporter gene expression was undetectable in a −108 to +45 promoter::GUS fusion, indicating that promoter sequences from −248 to −108 are essential for activity [40, 59].

The −248 to −108 region of the osmotin promoter contains sequences that bear similarity to the G-box elements [57], the AT-1 box [14] and GCCGCC elements [17, 36]. These sequences exist in many PR promoters [6, 16, 18, 21, 22, 33, 45, 52, 53]. The GCCGCC elements have been shown to be involved in specific interactions with nuclear factors [21, 36] and to confer ethylene responsiveness to heterologous promoters [36]. Deletion of the GCCGCC element results in the loss of ethylene responsiveness of PR promoters [6, 21, 33, 55]. However, it is not clear if G-box-like elements and AT-1 boxes in PR promoters interact specifically with nuclear proteins and function as transcriptional activators.

In this paper we present results of our dissection of the −248 to −108 region of the osmotin promoter to identify sequences of importance in this region. We demonstrate that the three conserved promoter elements lying in this region are protected against DNase I digestion by nuclear proteins and are involved in DNA protein interactions. Our results also indicate that these elements influence osmotin promoter activity and may do so by interacting with each other.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* cv. Wisconsin-38) suspension cells were maintained as described [3]. S-0 refers to cells grown in absence of salt and S-25 refers to cells adapted to growth on 428 mM NaCl. S-0 cells were harvested 4 days after subculture. S-25 cells were harvested 21 days after subculture. For ABA treatment, filter-sterilized ABA (100 μM) was applied to S-0 cells 3 days after subculture; cells were harvested 24 h later. Young leaves from 15-day old tobacco shoots in culture [40] were used for transient expression experiments.

Plasmids

The plasmids used for transient expression experiments are shown in Fig. 1. A *Bam* HI fragment containing the −46 to +8 region of the CaMV 35S promoter joined to the coding region of the GUS gene followed by the rubisco transcription terminator were cloned into the *Bam* HI site of pGEM-7zf(−) to give the plasmid 35S. Osmotin promoter fragment A (−248 to −108) and its derivatives were generated by PCR. The end-points of these DNA fragments relative to the transcription initiation site are shown in the figure. *Hind* III and *Bam* HI site sequences were added to the 5′- and 3′- end PCR primers, re-

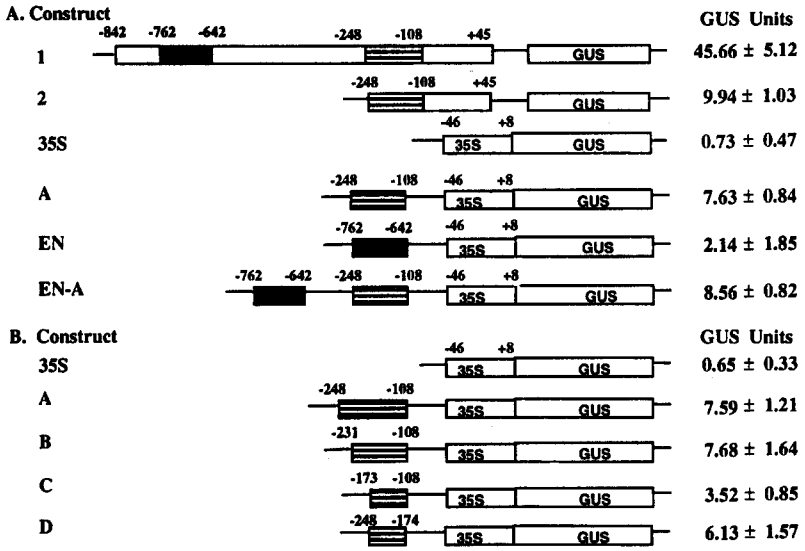

Fig. 1. Reporter gene expression of the osmotin promoter fragment::CaMV 35S::GUS fusion constructs in young tobacco leaves. Plasmid constructs carrying the GUS reporter gene fused to the test promoter sequence were introduced into tobacco leaves by microprojectile bombardment. GUS activities represent the mean of three bombardments ± standard deviation. **A.** Data showing that fragment A is sufficient for reporter gene expression. Constructs 1 and 2 represent chimeric truncated osmotin promoter::GUS fusions, (−842 to +45) and (−248 to +45) respectively in pBI201 [40]. 35S represents the minimal CaMV 35S promoter (−46 to +8)::GUS fusion in pGEM7z. A, EN and EN-A represent derivatives of plasmid 35S containing the osmotin promoter sequences, fragment A (−248 to −108) or/and fragment EN-1 (−762 to −642). **B.** Effect of deletions of fragment A on reporter gene activity. Construct 35S and A were as described above. B (−231 to −108), C (−173 to −108) and D (−248 to −174) represent derivatives of plasmid 35S containing the osmotin promoter sequences indicated. The osmotin promoter sequences were all cloned into the same cloning sites on construct 35S. The thin lines represent multiple cloning sites on the vectors.

spectively. The PCR products were cloned into the *Sma* I site on vector pGEM-7zf(−). The promoter fragments were then excised by digestion with *Xho* I and *Cla* I and inserted into the corresponding polylinker sites on plasmid 35S. Fragment EN-1 (−762 to −642), excised from the osmotin promoter by digestion with *Cla* I [40], was ligated into the *Cla* I site on plasmid 35S (construct EN). Construct EN-A, which contained both fragments EN-1 and A in the plasmid 35S, was made by excising fragment EN-1 from construct EN with *Sph* I and *Sst* I, blunting the ends with T4-DNA polymerase, and ligating into the blunted *Sph* I site of construct A. In all the plasmids, the osmotin promoter fragments were located immediately upstream of the minimal CaMV 35S promoter. All plasmid constructs were sequenced by the dideoxy chain termination method (Sequenase Version II, United States Biochemical). The plasmid pOD432, which was also used in the transient expression assays contained a luciferase gene driven by a CaMV 35S promoter, and was the kind gift of Dr David Ow.

Microprojectile bombardment

Tungsten particles (M-17, 1.0 μm) were used to deliver DNA using a Biolistics PDS-1000 He System (DuPont). Fifteen μg particles were coated with 5 μg DNA according to the manufacturer's instructions (BioRad). The DNA was a 1:1 (w/w) mixture of the test plasmid and pOD432. DNA delivery was optimized for particle size, distance between the tissue and particle discharge barrel and bombardment pressure. After bombardment, samples were kept in Petri dishes containing wet filter paper at room tem-

perature for 24 h before GUS enzyme assays. All experiments were repeated at least three times with three replicates each.

Enzyme assays

GUS and luciferase enzyme activities were measured as described by Raghothama *et al.* [40]. All GUS activities were normalized with respect to luciferase activities to account for variation caused by random particle distribution during bombardment [40]. A unit of GUS activity was defined as 1 nmol of 4-methylumbelliferone formed per mg protein per hour.

Gel mobility-shift assays

The oligonucleotides used in gel mobility-shift assays were chemically synthesized (Integrated DNA Technologies, Inc.). Complementary oligonucleotides (0.5 μg) were annealed in 20 μl T4-polynucleotide kinase buffer by successive 15 min incubations at 65 °C, 37 °C and 4 °C. The ds-oligonucleotide was treated with T4-polynucleotide kinase for 3 h at 37 °C and then with T4-DNA ligase at room temperature for 8 h, according to instructions provided by the manufacturer (Promega). The monomer, dimer, trimer and tetramer were separated on a 10% polyacrylamide gel, eluted, and ^{32}P-labeled by a fill-in reaction with Klenow DNA polymerase [43].

Nuclear extracts were prepared from tobacco suspension cells and young leaves as described [40]. The standard binding reaction (15 μl) contained 5,000 cpm of ^{32}P-labeled DNA fragments, 2 μg of poly(dI-dC), and 3 μg of nuclear protein, 25 mM Hepes buffer, pH 8.0, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 8% glycerol. The binding reaction was allowed to proceed at room temperature for 30 min. Reaction mixtures were electrophoresed on 8% polyacrylamide gels (acrylamide/bisacrylamide 29:1) in 0.5 × TBE buffer (1 × TBE is 89 mM Tris base, 89 mM disodium borate, 2 mM Na_2EDTA, pH 8.0). Gels were run at 10V per cm for 1 h, dried and exposed to X-ray film at −80 °C overnight. For gel mobility-super-shift assays, nuclear protein (3 μg) from S-25 cells were first incubated with 2 μl of anti-GF14 for 1.5 h. Then the standard binding reactions and electrophoresis were performed. Anti-GF14 antibody (a gift from Dr R.J. Ferl) was a monoclonal antibody raised against *Arabidopsis* GF14 protein [32].

DNase I footprinting

DNase I footprinting assays were performed essentially as described by Raghothama *et al.* [40]. Gel-purified fragment A labeled at one end (20000 cpm) and 20 μg nuclear proteins were used in a typical DNA-binding assay scaled up to 100 μl. DNase I treatment (Pharmacia, Sweden, 40 units per ml final) was for 3 min at room temperature.

UV cross-linking assay

UV cross-linking of DNA-binding proteins was performed essentially as described [2]. The DNA probes were prepared by annealing the primer 5′-TCTCAAGGATGCTTTGGCAAA-3′ (3 μg) to fragment A (50 μg) and extending the primer with Klenow DNA-polymerase in 10 mM Tris-HCl buffer pH 8.0 containing 10 mM $MgCl_2$, 50 mM NaCl and 0.5 mM each dATP, dGTP, 5-bromo-2′-deoxyuridine triphosphate and [α-^{32}P]-dCTP (50 μCi, 3000 Ci per mmol) in a volume of 20 μl. Gel purified DNA probe (5000 cpm) was incubated with nuclear extract (3 μg protein) for 30 min as described for gel mobility-shift assay. The microcentrifuge tubes containing samples were exposed to UV light (0.2 J) in a UV crosslinker (Stratagene). The samples were then subjected to DNase I digestion (1 unit per reaction) at room temperature for 15 min. The proteins cross-linked to DNA probe were separated on a 12% SDS-PAGE gel. After electrophoresis, the gel was dried and exposed to an X-ray film at −80 °C.

Results

Fragment A (−248 to −108) is sufficient to direct gene expression when fused to a minimal CaMV 35S promoter

Analyses of transient GUS expression in young tobacco leaves bombarded with chimeric constructs containing 5′ deletions of the osmotin promoter fused to GUS showed that fragment A (−248 to −108) was absolutely required for reporter gene expression [40]. Similar results have been obtained by analyses in transgenic tobacco seedlings [25]. In order to demonstrate that fragment A was sufficient to drive reporter gene expression, the chimeric promoter::GUS fusions shown in Fig. 1 were constructed and introduced into young tobacco leaves via microprojectile bombardment. In this system, fragment A fused to a minimal CaMV 35S promoter (−46 to +8) could initiate reporter gene expression to a level similar to that of a truncated osmotin promoter (−248 to +45). Fragment A fused to the minimal CaMV 35S promoter in the reverse orientation did not enhance reporter gene expression (data not shown). Osmotin promoter fragment EN-1 (−762 to −642), which has been shown to bind proteins in nuclear extracts derived from ethylene-treated leaves, ABA treated cultured cells and salt-adapted cultured cells [40], enhanced GUS activity very slightly. The difference between reporter gene activity observed with constructs 1 and EN-A could be due to the spacing between fragments EN-1 and fragment A. It is, however, more likely that promoter sequences between −642 and −248 also contribute to enhancement. This conclusion is supported by our earlier data which show that the reporter gene activities of chimeric osmotin promoter::GUS fusions increase continuously with promoter length between −248 and −1052 in this transient assay system [40].

We further analyzed the effect of deletion of 5′ and 3′ regions in fragment A on reporter gene expression (Fig. 1B). Deletion up to −231, which eliminated G-box like sequences, did not affect the reporter gene activity significantly. 5′ deletion up to −173 reduced reporter gene activity by about 54% and 3′ deletion up to −174 reduced activity by about 20%. This suggested that fragment A contains multiple sequences that interact and contribute towards gene expression.

Protein factors that can bind to fragment A are present in nuclear extracts of cells expressing the osmotin gene

Osmotin mRNA and protein accumulate to high levels in tobacco cells adapted to 428 mM NaCl (S-25) when compared with unadapted (S-0) tobacco cells [48]. Factors that can bind to fragment A were detected in nuclear extracts prepared from S-25 cells by gel mobility-shift assays (Fig. 2A). Consistent with the lower expression of the osmotin gene in S-0 cells [29, 48], this DNA-binding activity was significantly lower in nuclear extracts from S-0 cells. The DNA-binding activity in S-25 nuclear extracts was heat unstable and proteinase K-sensitive, indicating that it was proteinaceous in nature. The DNA-binding proteins in S-25 extracts bound to fragment A in a sequence-specific manner as shown by competition experiments (Fig. 3). Poly[dA-dT] (up to 2 μg) also did not compete for binding with labeled fragment A (data not shown).

Fragment A-binding activity was found in nuclear extracts of S-0 cells treated with 100 μM ABA (Fig. 2B). ABA activates osmotin gene transcription in S-0 cells [34]. Fragment A-binding activity was also found in nuclear extracts of young cultured tobacco leaves (Fig. 2C) where the osmotin gene is expressed constitutively (Y. Xu, unpublished data). These results indicated that expression of osmotin gene is associated with specific interaction of fragment A with nuclear proteins.

Mapping of protein-binding sites by DNase I footprinting

DNase I footprinting assays were carried out to locate the protein binding sites on fragment A. As shown in Fig. 4, several regions on the 5′-end-

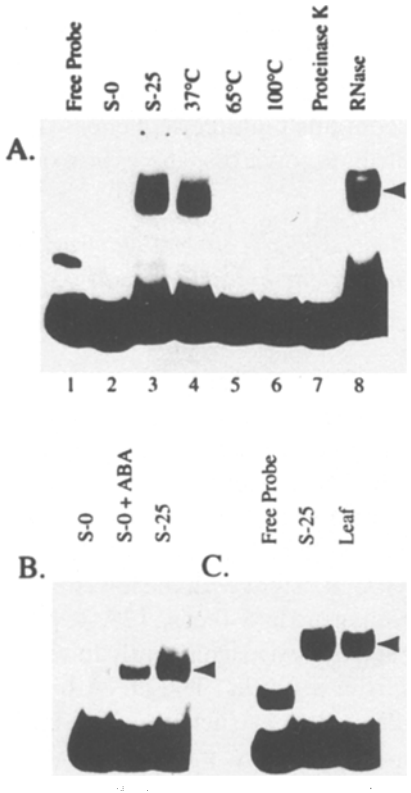

Fig. 2. Gel mobility-shift analysis of the binding of nuclear protein factors to fragment A. **A.** Effect of protein denaturing treatments on DNA-protein interactions. Lane 1, ^{32}P-labeled fragment A with no added nuclear extract; Lane 2, nuclear extract from S-0 cells added (3 μg protein); Lanes 3–8, nuclear extract from S-25 cells added (3 μg proteins) except in lane 3, all extracts were subjected to the indicated treatment prior to the binding reaction. **B.** Induction of DNA-binding proteins by ABA and NaCl. The labeled fragment A was incubated with nuclear proteins (3 μg) from S-0 cells (lane 1); 100 μM ABA-treated S-0 cells (lane 2), and S-25 cells (lane 3). **C.** Relative abundance of DNA-binding proteins in different cell types. Lane 1, free probe; Lane 2, nuclear extract from S-25 cells added (3 μg proteins); Lane 3, nuclear extract of young leaves from shoot culture added (3 μg proteins). The bound probe is indicated ($\surd$).

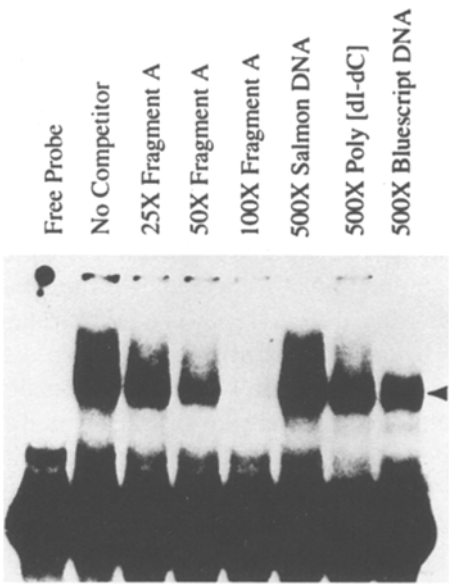

Fig. 3. Gel mobility-shift analysis showing the effect of unlabeled competitors on the binding of protein factors to fragment A. Left lane: ^{32}P-labeled fragment A with no added nuclear extract. All other lanes represent binding reactions in the presence of nuclear extract from S-25 cells (3 μg proteins). The indicated mass excess of unlabeled competitor DNAs were added to the standard binding reaction. Denatured salmon sperm DNA and pBluescript DNA were digested with *Sau* 3A and *Hha* I before use. The bound probe is indicated ($\surd$).

labeled fragment were protected from DNase I digestion by S-25 nuclear proteins. The same regions were also protected when nuclear extracts from 100 μM ABA-treated S-0 cells were used (data not shown). The protein binding regions of osmotin promoter fragment A that were detected by DNase I footprinting included the sequence 5′-AATTATTTTATG-3′ (−172 to −183, AT sequence) which is similar to the consensus AT-1 box, 5′-A/GA−/TTATTTTTAT/AT/C-3′ [14] and the sequence 5′-TAAGA/CGCCGCC-3′ (−153 to −163, −148 to −138, PR sequence) which is an ethylene-responsive element present in a number of PR-gene promoters [16, 21, 36]. Protein binding to the 5′-end sequences up to −213 was confirmed by DNase I footprinting using 3′ end-labeled fragment A (data not shown). This region included the sequence 5′-CAAGTGTCACGTT-3′ (−245 to −233, G sequence) which has sequences resembling the consensus G-box element 5′-CACGTG-3′ [57].

Complementary synthetic oligonucleotides containing either the G, AT, PR or protected promoter sequences between −231 and −213 (termed NC) were polymerized. The dimer, trimer and tetramer of each oligonucleotide was used for gel mobility-shift assays (Fig. 5). The NC oligonucleotide was unable to bind protein factors in S-25 nuclear extracts. The G, AT and PR

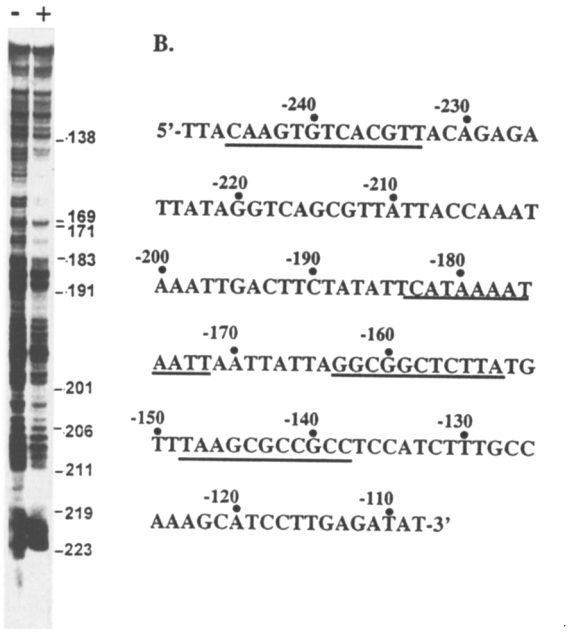

Fig. 4. Mapping of protein binding regions on fragment A by DNAse I footprinting. **A.** Shown is a DNase I footprinting reaction of fragment A after a binding reaction with S-25 nuclear extract. The lanes denote DNase I digestion reaction performed on the 5'-end-labeled fragment A in the absence (−) or presence (+) of nuclear extract. The position of the bases are indicated. **B.** Diagram showing the location of conserved elements on fragment A. The conserved sequences G (−245 to −233), AT (−183 to −172) and PR (−163 to −153, −148 to −138) which were protected from DNase I digestion are underlined.

oligonucleotides showed protein binding activity. The binding of protein factors to the labeled oligonucleotide tetramers could be competed by an excess of the unlabeled tetramer but not by an excess of poly dI-dC or *Hin*d III plus *Hae* I digested pBluescript DNA (data not shown). The transient gene expression data supported the existence of more than one sequence in fragment A contributing to osmotin gene expression. The DNA-protein interaction data suggest that G, AT and PR sequences may be involved in osmotin gene expression. The transient expression data (Fig. 1B) when combined with these DNA-protein interaction data suggest that AT and PR sequences may be involved to a greater extent in osmotin gene expression in microprojectile-bombarded cultured tobacco leaves.

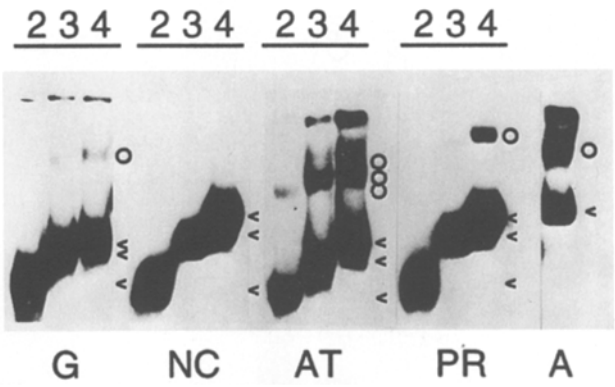

Fig. 5. Gel mobility-shift assays using synthetic oligonucleotides. The synthetic oligonucleotides used were:
G: ATGCTTACAAGTGTCACGTTAC (−248 to −231)
AATGTTCACAGTGCAATGTACG
NC:ATGCAGAGATTATAGGTCAGCG (−231 to −213)
TCTCTAATATCCAGTCGCTACG
AT: ATGCTTCTATATTCATAAAATA (−193 to −173)
AAGATATAAGTATTTTATTACG
PR: ATGCTATTAGGCGGCTCTTATGTT (−168 to −149)
ATAATCCGCCGAGAATACAATACG
Gel purified 2-mer, 3-mer and 4-mer of the oligonucleotides were end-labeled by a Klenow fill-in-reaction and used in a standard gel mobility-shift assay with S-25 nuclear extract (3 μg proteins). The position of free probe (√) and protein-bound probe (O) are shown. The degree of polymerization (2, 3, 4) is indicated on the figure. Lane A is a mobility-shift assay performed with fragment A.

Analysis of protein factors associated with fragment A

UV cross-linking assays were conducted to determine the number of proteins involved in the protein complex that specifically bind to fragment A. In nuclear extracts from S-25 cells and young cultured tobacco leaves, four proteins with approximate molecular masses of 42, 40, 29, and 28 kDa were cross-linked to fragment A (Fig. 6). Similar data was also obtained using ABA-treated S-0 cells (data not shown).

The G-box element of *Arabidopsis Adh* gene promoter is known to interact with a protein factor, GF14, a 14-3-3 protein homolog [32]. S-25 nuclear extracts contain a 29 kDa protein which cross-reacts with anti-GF14 antibodies. Using gel mobility-super-shift assays with anti-GF14 antibodies we have previously demonstrated that

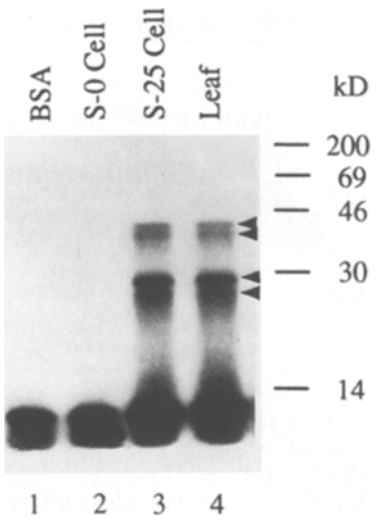

Fig. 6. UV cross-linking assay showing the proteins bound to fragment A. Shown is an autoradiogram of the cross-linked proteins separated by SDS-PAGE. The UV cross-linking assays were performed as described in Methods using nuclear extracts (3 μg proteins) of S-0 cells, S-25 cells and young leaves from shoot culture. Bovine serum ablumin (BSA, 3 μg) was used as a negative control. The positions of cross-linked proteins are marked (√).

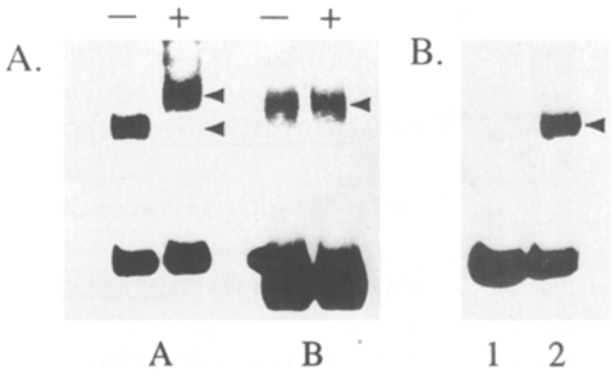

Fig. 7. 14-3-3-like proteins are associated with fragment A. **A.** Determination of the binding site of 14-3-3-like proteins on fragment A. Shown are the results of gel mobility-shift assay performed with S-25 nuclear extract (3 μg proteins) and osmotin promoter fragments A (−248 to −108) or B (−231 to −108) in the absence (−) or presence (+) of anti-GF14 antibody. **B.** Interaction of recombinant tobacco 14-3-3 protein with fragment A. Shown are results of a standard gel mobility-shift assay performed with purified recombinant tobacco 14-3-3 protein (3 μg, lane 1) and S-25 nuclear extract (3 μg protein, lane 2). Recombinant tobacco 14-3-3 protein was purified as described by Chen *et al.* [12]. The positions of the bound probe are marked (√).

S-25 nuclear extracts contain 14-3-3-like proteins which are present in the protein complex bound to fragment A [12]. Here we further demonstrate that these proteins are specifically associated with the G-box sequence on fragment A based on gel mobility-super-shift assays (Fig. 7A). When S-25 nuclear extract was incubated with anti-GF14 antibody before the binding reaction, a super-shifted band was observed when using fragment A as a probe. This super-shifted band was not found when fragment B (−231 to −108) lacking the G-box like sequences was used in binding assays. Recombinant tobacco 14-3-3-like protein [12] was not able to bind directly to DNA fragment A as indicated by the gel mobility-shift assays (Fig. 7B). These results are consistent with the result obtained with the *Arabidopsis Adh* gene promoter [32] and indicate that in addition to the four proteins detected by cross-linking, 14-3-3-like proteins are associated with fragment A. The results also show that the imperfect G-boxes on fragment A are capable of interacting with 14-3-3 like proteins.

Discussion

We have demonstrated that the osmotin promoter fragment A (−248 to −108) is sufficient to direct reporter gene expression when fused with a minimal CaMV 35S promoter. These results confirm and extend the results of our earlier observations [40]. Osmotin gene expression appears to require the binding of nuclear proteins to fragment A (Fig. 2). It is difficult to find conditions wherein the osmotin gene is not expressed, with the exception of S-0 cells in early stages of the growth cycle that do not have detectable levels of osmotin mRNA or protein. Nuclear extracts derived from these cells did not show any protein binding activity with fragment A.

There appears to be a similarity between DNA-protein interactions that occur on fragment A in the three situations examined, S-25 cells, ABA-treated S-0 cells, and young cultured tobacco leaves. Proteins of the same molecular weight were cross-linked in all three instances. The same DNase I footprinting pattern also was seen using

nuclear extracts from S-25 and ABA-treated S-0 cells. It is tempting to speculate that in all instances when the osmotin gene is expressed, the same four proteins are bound to fragment A at the same promoter elements. The entire DNA-protein complex on the promoter fragment can, however, consist of other proteins, such as the 14-3-3 proteins, that do not directly interact with DNA and are not cross-linked to it by UV. Quantitative differences in expression level can then be envisaged as the results of interactions of the four DNA-bound proteins with the other proteins or due to covalent modification of some proteins on the complex.

The G, AT and PR sequences on fragment A, which lie in regions protected in the DNase I footprinting assay and can interact specifically with nuclear protein factors, bear close similarity to previously recognized promoter elements [14, 21, 36, 57]. Examination of the sequence of a number of PR gene promoters [6, 9, 16, 18, 19, 22, 35, 38, 42, 45] shows that these conserved sequences occur in many PR promoters, and often in promoter regions shown by deletion analysis to be necessary for response to signal molecules. For example, promoter sequences (−607 to −321) which were shown to be necessary for TMV and salicylic acid induction of an acidic extracellular glucanase of *Nicotiana tabacum* by deletion analysis, contain an AT-1-like sequence and G-box-like sequence [22]. Similar studies have determined that the −788 to −345 region of a *N. tabacum* basic endochitinase promoter and the −422 to −195 region of the bean chitinase CH5B contained sequences that potentiate high level of expression and ethylene-induced expression, respectively [6, 18]. Two PR sequences and five AT-1-like sequences lie in the −788 to −344 region of the *N. tabacum* chitinase promoter. One PR sequence and one AT sequence are present in −422 to −195 bp region of the bean chitinase CH5B promoter. Other chitinase promoters such as tobacco CH17 [45], and *Arabidopsis* A2 [42] also have PR sequences and AT sequences in their promoters. However, it is not known if these motifs are in a region of the promoter that has any activity.

The sequence of the PR-1b promoter that confers ethylene responsiveness was inferred to lie in the −213 to −141 region by deletion analysis [17, 33]. It contains both G-box and PR sequences. Although this entire region had nuclear protein binding activity, an oligonucleotide covering the −201 to −178 sequence (containing PR sequence and G-box) had no protein binding activity. An oligonucleotide corresponding to promoter sequence −179 to −154, that had no recognizable *cis* element, had binding activity. Therefore there is as yet really no clear evidence that the PR sequence or the G-box have protein binding activity or function in the activity of the PR-1b promoter. AT sequences are also present on the PR-1b promoter, but upstream from the minimal element.

All these data show that there exist common elements in PR gene promoters but evidence for their involvement in DNA-protein interactions that lead to PR gene expression is scarce. We have demonstrated here that three conserved promoter elements (G, AT, and PR) which lie within the minimal promoter region that is required for osmotin promoter activity, also have protein binding activity. Although we have no evidence that the AT sequence can by itself direct gene expression, a promoter fragment containing the AT sequence along with G-box sequence was able to direct GUS expression when fused with a minimal CaMV 35S promoter (Fig. 1B). AT sequences have been reported to stimulate transcription in yeast [11, 50]. Their role in plant promoters, however, is not clearly understood. They occur in a number of PR promoters although not necessarily in the minimal responsive region. An AT sequence element from the promoter of French bean phaseolin, the small subunit of pea ribulose diphosphate carboxylase (*rbcS-3A*) and soybean heat shock protein (Gmhsp17.5E) were all found to stimulate transcription when fused to a minimal CaMV 35S promoter [8, 13, 27]. However, Castresana *et al.* [10] identified an AT sequence of *Nicotiana plumbaginifolia* chlorophyll *a*/*b*-binding protein (*cabE*) as a negative regulatory element. An 11 bp AT-rich region of the parsley PR-2 promoter has been identified as the

cis element required for elicitor-mediated expression [26]. Clones encoding transcription factors binding to this element have been isolated from parsley and *Arabidopsis thaliana* and appear to encode homeodomain proteins [26]. The parsley PR-1 and PR-2 proteins to not seem to be related to the tobacco PR-1 and PR-2 proteins [30]. The AT sequence on osmotin promotor fragment A had a positive effect on reporter gene expression.

We have since identified mutations in the AT and PR sequences that cause loss of DNA-protein interactions *in vitro* (P. Xu, unpublished). By introducing these mutations in the context of fragment A, it should be possible to further delineate the role of these sequences in the osmotin promoter.

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