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Identification of *cis*-acting elements involved in the regulation of the pathogenesis-related gene STH-2 in potato

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Abstract

We have characterized a genomic clone containing the potato pathogenesis-related genes STH-2 and STH-21. The two genes are found 4 kb apart on the same chromosome and their sequences are highly similar. They present the same transcriptional orientation and are both interrupted by a single intron. A chimaeric gene consisting of 1015 bp of 5′-flanking sequence and part of the first exon of STH-2 fused to the bacterial β-glucuronidase gene was highly-expressed in tubers of transgenic potato plants after wounding and elicitor treatments. The levels of activity observed in these transgenic plants parallel those observed for the accumulation of STH-2 mRNAs under similar conditions. This indicates that *cis*-acting elements necessary for the proper activation of the gene are present within 1 kb of 5′-flanking sequences. Functional analysis of 5′ deletions of the STH-2/GUS constructs by transient expression in leaf protoplasts revealed the presence of an upstream regulatory sequence between −135 and −52 which contains a TGAC motif, and a possible negative regulatory region between −52 and −28. A factor present in nuclear extracts of wounded potato tubers was found to bind specifically to nucleotides located between −135 to −105, suggesting that this region contains important *cis*-regulatory elements.

Introduction

Pathogen infection and wounding are two important stress conditions encountered by plants. In both cases, plants respond by altering the expression of specific genes, an event which often results in profound modifications in primary and secondary metabolism. In numerous plant-microbe interaction systems, a consensus of events contributing to the resistance of the plant has now been identified [see 16, 23 for recent reviews]. In the potato, typical hypersensitive response symptoms appear when the leaves or tubers are inoculated with an incompatible (avirulent) race of the late blight fungus *Phytophthora infestans* (Mont.) de Bary, or when the plant is treated with an elici-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers M29041 (STH-2) and M29042 (STH-21).

tor such as arachidonic acid. The hypersensitive response is characterized by a coordinated death of cells immediately surrounding the point of pathogen ingress. How it is controlled and mediated is unclear, but the resulting zone of dead cells is thought to inhibit further growth of the pathogen [34]. This response is also accompanied by changes in various metabolic activities, including the synthesis of sesquiterpenoid phytoalexins [8], chitinase and β-1,3 glucanases [22] as well as phenylalanine ammonia-lyase, 4-coumarate:CoA ligase [15] and alcohol dehydrogenase [7, 31]. Many genes coding for proteins of unknown function are also induced in potato upon infection, elicitor treatment or wounding [25, 41, 43]. One such group of genes is exemplified by the STH-2 gene family, which was shown to be induced rapidly in potato during the hypersensitive response [28, 29]. STH-2 protein accumulates following elicitation or infection in tuber, stem, petiole and leaf tissue [6, C.P. Constabel and N. Brisson, in preparation]. Significant levels were also detected after wounding. Whether STH-2 protein is required for resistance to pathogens is not yet clear; however a low concentration of spores induced accumulation of STH-2 protein faster and to higher levels in the incompatible (host-resistant) than in the compatible (host-susceptible) interaction [6].

The function of the STH-2 gene product is unknown. Sequence data comparisons do not point to any biological activity. However, STH-2 homologous genes were also described from infected pea endocarp [14] and elicited parsley and bean cell suspensions [40, 45]. Recently a related gene from a monocot plant family was cloned from mechanically isolated and cultured asparagus cells [46]. STH-2 homologous genes have also been found to be expressed during late pea embryogenesis [1], in auxin-starved soybean cell cultures [9], as well as in birch pollen [3]. The presence of homologous genes activated by infection or elicitation in both monocot and dicot plant families suggests that STH-2 is important in the plant disease response.

We have reported the sequence of the gene corresponding to the pSTH-21 cDNA clone [30]. In the present study we report the isolation and characterization of the gene corresponding to the pSTH-2 cDNA clone. We show that the STH-21 and STH-2 genes are found closely together on the same chromosome and that their structure is highly similar. About 1 kb of 5′ flanking DNA is sufficient to direct wound and elicitor induction of the STH-2 gene in transgenic plants and a short 30 bp region of this flanking DNA appears to play an especially important role for the expression of this gene.

Materials and methods

Materials

DNA modification enzymes were purchased from New England Biolabs, Beverly, MA. Arachidonic acid was purchased from Sigma, St.-Louis, MO. The Sequenase sequencing kit was purchased from U.S. Biochemical, Cleveland, OH. Cellulysin and macerase were from Calbiochem, LaJolla, CA. Ancymidol was a gift from Elanco Products, Indianapolis, IN. pBI101 and pBI221 vectors were purchased from Clontech Laboratories, Palo Alto, CA.

Isolation and characterization of genomic clones

The construction of a λEMBL3 potato genomic library has been described previously [4]. The library was screened with the pSTH-2 cDNA insert [29] radiolabelled by the random primer method [13] and positive clones were purified by three rounds of plating and screening. Phage DNA was isolated from a selected clone and the 12 kb insert was mapped by the partial digestion method of Smith and Birnstiel [39]. DNA fragments were subcloned in the pBluescript plasmid vector (Stratagene, San Diego, CA) and the sequence was determined by the dideoxy chain termination method [36]. The transcription start site was determined as described by Wu *et al.* [48] by primer extension of mRNAs isolated from 24 h elicitor-treated potato tuber discs. The 16-mer

primer used in these experiments was located at position +79 to +94.

Potato transformation and assay for GUS activity

A *Hin*d III fragment spanning the region from −1015 to +145 of the STH-2 gene was modified by ligation of a 10-mer *Hin*d III to *Bam* HI adaptor (5′-AGCTGGATCC-3′). The modified fragment was inserted into the *Bam* HI restriction site in the transformation vector pBI101 which contains the *Escherichia coli uidA* (β-glucuronidase, GUS) gene followed by the nopaline synthase 3′ terminator region [18, 19]. This placed the STH-2 coding sequence in phase with the GUS coding sequence. The STH-2/GUS construct was then introduced into the disarmed *Agrobacterium tumefaciens* strain LBA4404 by the triparental mating procedure [12]. Potato plants (*Solanum tuberosum* L. cv. Désirée) were transformed using a modified leaf disc co-cultivation method [10, 17]. Mini-tubers were prepared by placing nodes of *in vitro* grown transgenic Désirée potato plants on solid (0.6% agar) MS medium [33] supplemented with 80 g/l sucrose and 3 mg/l ancymidol. Mini-tubers were grown in the dark at 20 °C for one month and used immediately after harvesting. The mini-tubers (ca. 6 mm in diameter) were cut in halves, placed on a moistened sterile Whatman 3MM paper in a small Petri dish and the cut surface treated with 5 μl of water (wound response) or 5 μl of an emulsion of arachidonic acid (1 μg/μl) (elicitor treatment) for 24 h. Unsliced mini-tubers from untransformed plants were used as controls. The mini-tubers were ground at 4 °C in extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% v/v Triton X-100, 0.1% Sarkosyl, 15 mM 2-mercaptoethanol, [19], the extracts cleared by centrifugation, and the protein concentration adjusted to 0.1 mg/ml. GUS assays were performed in 50 μl of extraction buffer with 1 mM 4-methylumbelliferyl glucuronide (MUG) as described [19]. Fluorometric measurements were performed on a Hoefer Scientific DNA Fluorometer.

STH-2/GUS constructions

The −1015 to +145 STH-2 modified fragment was inserted at the *Bam* HI site of the promoterless pBI201 vector which was obtained by deletion of the CaMV 35S promoter from the pBI221 vector. Deletions in the 5′-upstream region of STH-2 were generated by using useful restriction sites (*Eco* RI, −670; *Spe* I, −549; *Eco* 47 III, −281; *Xmn* I, −179; *Xba* I −135 and −52), ligation of synthesized fragments to an existing deletion (−80) and PCR amplification (−27 and −5). The CaMV 35S minimal promoter [2] was excised as an *Eco* RV/*Bam* HI fragment (−90, +5) from the pBI221 plasmid. The DNA spacer was a 1 kb fragment of mitochondrial DNA from *Allomyces macrogynus*

Protoplast isolation and electroporation

Leaf mesophyll protoplasts were isolated from 6-week-old *in vitro* grown potato plants cv. Désirée. The protoplast isolation procedure and culture media are as described by Magnien [27], except for the enzymatic solution which contained 2% w/v cellulysin and 0.5% w/v macerase. Electroporation of protoplasts was conducted with a home-made capacitor discharge system, using the disposable electroporation chambers (0.4 cm) of the Cell-Porator System of Gibco-BRL (Gaithersburg, MD). The electrical pulse was delivered from a 1000 μF capacitor charged at 185 V. Pulses from the electroporator were delivered to 320 μl of protoplasts (1×10^6 protoplasts/ml) to which was added 160 μl of a solution containing 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 335 mM mannitol and 50 μg of CsCl-purified supercoiled plasmid DNA (1 μg/μl). After electroporation the protoplasts were left on ice for 10 min and then transferred to 3.5 ml of medium. They were incubated at room temperature for 18 h in the dark, harvested by gentle centrifugation and lysed by vortexing in the GUS assay medium [19]. Protein content was determined using the BioRad Bradford protein assay. GUS activity was measured according to Jefferson *et al.*

[19]. Each construction has been assayed by electroporation with 3 to 10 batches of protoplasts. The results shown in Figs. 4 and 5 have been obtained with two batches of protoplasts prepared at different times, and each construction has been electroporated three times with each batch of protoplasts. Expression levels are expressed relative to the value of the −1015 to + 145 GUS construction which was used as an internal control.

Preparation of crude nuclear extracts

Treatment of potato tubers with arachidonic acid (elicitor response) and water (wounding response) was performed as described previously [28]. The preparation of nuclear extracts was carried out essentially by the method of Willmitzer and Wagner [47]. The tuber discs treated for 17 h and freshly cut tubers were homogenized in a blender in 2 ml/g ice-cold buffer C (0.25 M sucrose, 10 mM NaCl, 10 mM MES-NaOH pH 6.0, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM 2-mercaptoethanol and 0.2 mM phenylmethylsulphonyl fluoride (PMSF)) containing 0.6% Nonidet P40 and 0.1% bovine serum albumin (BSA). The homogenate was filtered through three layers of Miracloth and one 60 μm nylon membrane then centrifuged for 5 min at 4000 × *g*. The crude nuclear pellet was resuspended in 1/10 volume buffer C containing 0.6% Nonidet P40, loaded on a 50%/88% Percoll step gradient and centrifuged for 15 min at 4000 × *g* in a swinging-bucket rotor. Material at the interface was collected and diluted with buffer C. The nuclei were pelleted by centrifugation at 4000 × *g* for 5 min. Nuclear proteins were prepared from this crude nuclear pellet by resuspension and sonication in 0.5–1 ml extraction buffer (420 mM KCl, 20 mM HEPES-NaOH pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol). The mixture was incubated for 1–2 h on ice, and nuclear debris were removed by centrifugation for 15 min at 15000 × *g*. Extracts containing nuclear proteins were dialysed for 3 h against 100–200 volumes of extraction buffer containing 50 mM KCl. Aliquots were frozen and stored at −80 °C.

Probe and competitor DNA preparation

All probes were labelled at the 3′ end using the Klenow fragment of DNA polymerase I. Total incorporation of radioactivity (usually 10^4–10^5 cpm/ng of DNA) was assayed on DE81 paper. The STH-2 probe and competitor DNA fragments spanning the region −135 to −52 were isolated by preparative gel electrophoresis. The AP-1 DNA element, which interacts with the proteins c-Jun and c-Fos, was isolated from a plasmid containing several copies of the element [11]. The plasmid pBluescript (pBS) was obtained from Stratagene.

Gel retardation assays

Binding reactions were carried out at 22 °C for 30 min in a total volume of 30 μl containing 20 mM HEPES-NaOH pH 7.9, 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 6% glycerol, 2 μg poly(dIdC), 15 μg of crude nuclear extract and the labelled DNA fragment. Competition reactions were conducted with various competitors added in the molar ratios described in the text. After incubations, reactions were loaded onto 6% polyacrylamide gels (30:0.8, acrylamide/bisacrylamide in 50 mM Tris-HCl pH 8.0, 380 mM glycine, 2 mM EDTA), which had been pre-run at 8 V/cm for 1 h. Electrophoresis was performed under the same conditions. Gels were blotted on Whatman 3MM paper, dried and exposed to X-ray films.

Results

Structure of the STH-2 and STH-21 genes

A genomic library in λEMBL3 was screened with the cDNA insert from pSTH-2 [29] and a positive clone containing a 12 kb insert was charac-

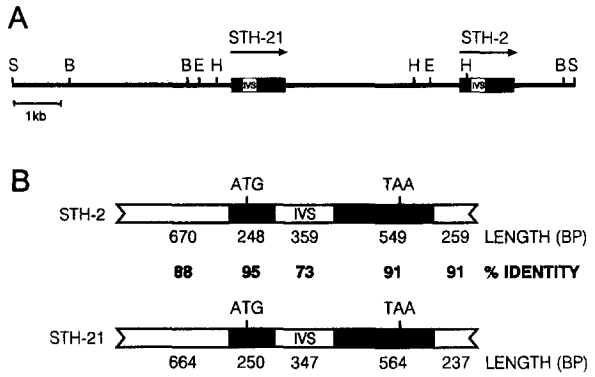

Fig. 1. A. Organization of two members of the STH-2 gene family on a 12 kb potato genomic clone. B. Comparison of the STH-2 and STH-21 genes. The black boxes represent exon sequences and IVS is the intervening sequence. The percentage identity was calculated using the Align program [24]. Arrows indicate the direction of transcription. S, *Sal* I; B, *Bam* HI, E, *Eco* RI; H, *Hin*d III.

terized by partial digestion mapping. Regions of the insert hybridizing to the pSTH-2 cDNA insert were subcloned and analyzed by sequencing. The results indicated that two highly similar genes separated by approximately 4 kb were present on the genomic clone (Fig. 1A). The sequence of one of these genes (STH-21) has already been reported [30] and was shown to correspond perfectly (100% match) to the sequence of the previously characterized pSTH-21 cDNA [29]. The sequence of the second gene has now been determined and is presented in Fig. 2. The sequence of the exons, including the untranslated regions, shows 100% similarity with the sequence of the previously characterized pSTH-2 cDNA [29]. The STH-2 and STH-21 genomic sequences therefore likely correspond to active genes.

Both STH-2 and STH-21 contain a single short intervening sequence interrupting the coding regions at amino acid 57. Figure 1B shows that both 5′-upstream and 3′-downstream regions are highly-conserved between the two genes. The 5′-

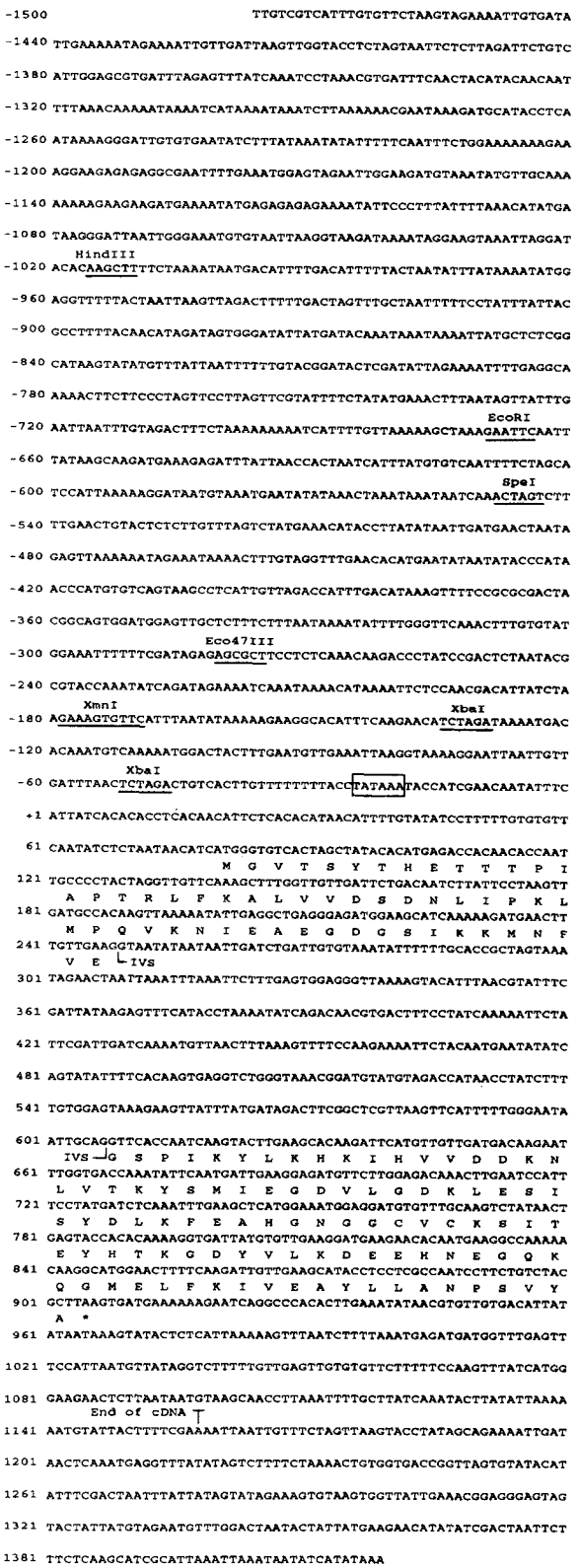

Fig. 2. Nucleotide and deduced amino acid sequences of the STH-2 gene. The restriction sites used to create the deletions analysed in Fig. 4 are indicated. The TATA element is boxed. The transcription start site is the +1 nucleotide.

upstream sequence similarity extends throughout 660 bp and then decreases abruptly. The 88% identity in nucleotide sequence in this region is almost as high as that observed between the coding regions. The nucleotide sequence similarity is also very high (91%) for at least 237 bp downstream of the poly(A) addition site. Multiple transcription start sites have been identified by reverse transcription of polyadenylated RNA from elicitor-treated tuber discs, using an oligonucleotide primer complementary to nucleotides + 79 to + 94 (data not shown). The major and most upstream site was designated as the + 1 nucleotide (Fig. 2). A putative TATA box element is located 25 bp upstream of the + 1 nucleotide.

STH-2 expression in transgenic potato plants

To determine whether *cis* elements required for the induction of STH-2 by elicitor treatment and by wounding were present in the 5′-flanking region, the sequence from −1015 to + 145 was fused translationally to the *E. coli uidA* gene (β-glucuronidase, GUS) and transgenic potato plants were produced. This translational fusion encompassed all the STH-2 5′-untranslated leader sequence plus 22 amino acids from the first exon of STH-2, and 8 amino acids derived from the polylinker and the GUS 5′-untranslated leader. Figure 3 shows that, for ten independent transgenic potato plants harboring the STH-2/GUS gene fusion, the level of activity in unsliced mini-tubers was comparable to the level observed in tubers from untransformed plants, indicating that the gene is not expressed to a significant level in intact mini-tubers. Slicing the mini-tubers, however, lead to a 3-fold increase in activity, while treatment with arachidonic acid elicitor resulted in an approximately 40-fold induction. The ratios of activities in intact versus wounded tubers (3-fold increase) and wounded versus elicitor-treated tubers (13-fold increase) were comparable for all clones. These results indicate that the region from −1015 to + 145 is sufficient to confer wound and elicitor induction of the gene.

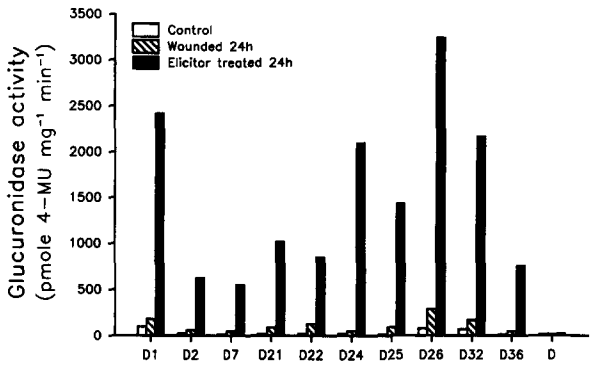

Fig. 3. Comparison of β-glucuronidase activity induced by wounding or elicitor treatment of transgenic potato plants. The STH-2 region from −1015 to + 145 was fused to the β-glucuronidase reporter gene and introduced in potato via *A. tumefaciens* transformation. Mini-tubers from 10 different transgenic plants were cut in half and treated with water (wounding) or arachidonic acid (elicitor treatment) for 24 h. Unsliced mini-tubers served as controls. D = untransformed mini-tubers.

Deletion analysis of the STH-2 promoter

The −1015 to + 145 STH-2 region was further analyzed in transient expression assays to determine if specific DNA sequences having regulatory functions could be identified. Figure 4 shows that in potato leaf mesophyll protoplasts, the −135 to + 145 region confers the same level of activity as the region extending to −1015. This suggests that no regulatory sequences important for the activity of the gene (as assayed in this transient expression system) are present in the −1015 to −136 region. However, deletions −105, −80 and −52 showed a 3-fold decrease in GUS activity when compared to the −135 deletion. Deletion −27 consistently showed higher reporter gene activity than the −52 deletion. Deletion −5 showed a very low level of GUS activity, which was only about twice the background level observed with the promoterless gene cassette pBI201. These results suggest that an upstream activating sequence (UAS) is present in the −135 to −105 region. The significant decrease in activity observed when the −27 to −5 region is removed indicates that an important regulatory element is present in this region. This element could be a TATA box, as

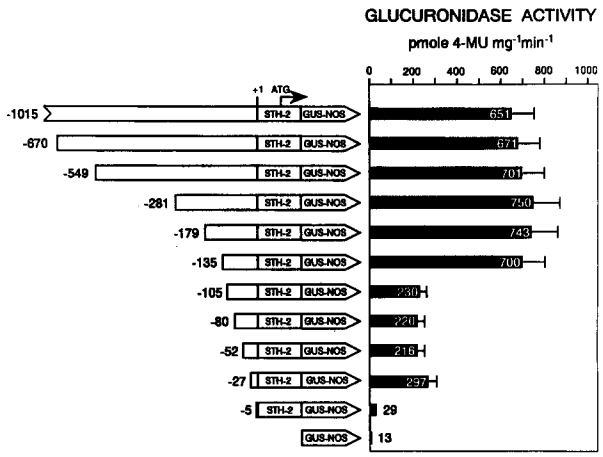

Fig. 4. Effect of 5′ promoter deletions on the level of GUS activity. Different deletions of the STH-2 gene 5′ flanking region were fused to the GUS reporter gene and analysed for their activity by electroporation in potato leaf mesophyll protoplasts. Means and standard deviations are from six electroporated replicates.

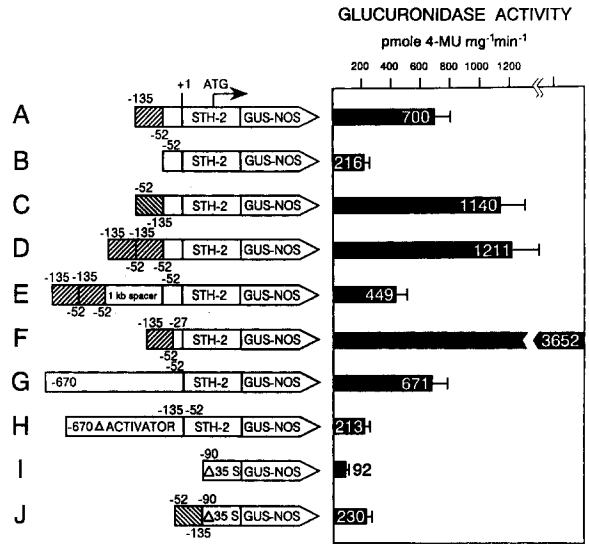

Fig. 5. Effect of the −135 to −52 region on reporter gene activity. The region from −135 to −52 (hatched box) was further analyzed by electroporation in potato leaf mesophyll protoplasts. The numbering defines the borders of the DNA fragments and their orientation. The Δ35S is the CaMV 35S minimal promoter (−90 to +5). Means and standard deviations are from six electroporated replicates.

suggested by the presence of the sequence TATAAATA at positions −25 to −18.

Characterization of the STH-2 upstream activating sequence

The function of the putative upstream activating sequence present between nucleotides −135 and −52 was further characterized in the transient expression system. Figure 5 indicates that this sequence could still increase transcription when placed in the inverse orientation (Fig. 5C), with activity levels higher than with the sequence placed in the correct orientation (Fig. 5A). The duplication of this sequence in the sense orientation (Fig. 5D) resulted in almost twice the activity observed with a single element. The ability to activate transcription at a distance was also investigated by inserting a 1 kb DNA spacer between two UAS and the −52 deletion (Fig. 5E). This led to a two-fold increase in reporter gene activity compared to the −52 deletion (Fig. 5B), but to a three-fold decrease in activity when compared to the same construct without the 1 kb spacer (Fig. 5D). The 1 kb DNA spacer had no effect on the activity of the −52 deletion in the absence of the UAS (not shown). An internal deletion removing the UAS from the STH-2 promoter while keeping the rest of the sequence intact (Fig. 5H) had the same effect as deleting all the promoter sequence up to nucleotide −52 (compare Fig. 5G, H and B), suggesting that the UAS functions independently and as a complete unit. This is supported by the results showing that an inverted STH-2 UAS retains its enhancing activity when positioned upstream of the CaMV 35S minimal promoter (Fig. 5J). Finally, the insertion of the UAS upstream to the −27 deletion had a pronounced effect on reporter gene activity (Fig. 5F). This gene fusion showed a 5-fold increase activity over the −135 deletion (Fig. 5A), suggesting that the region from −52 to −28 has the properties of a negative regulatory element.

Gel retardation

In order to identify protein factors interacting with the STH-2 UAS, we characterized DNA-protein

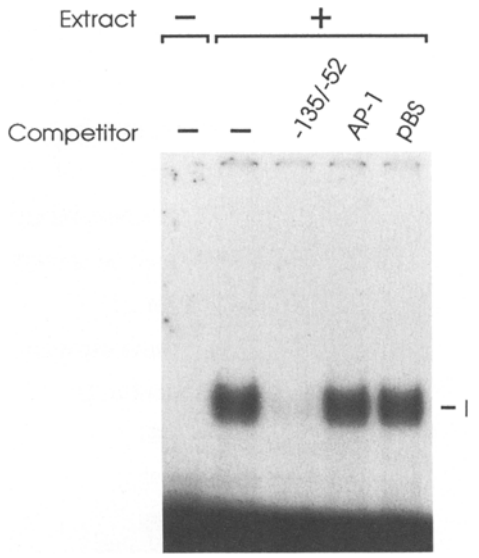

Fig. 6. Assay of STH-2 specific binding activity in the potato nuclear extract. The probe (STH-2, fragment −135 to −52) was incubated in the absence (−) or the presence (+) of a nuclear extract prepared from 17 h elicited tuber disks. A forty-fold molar excess of specific competitor DNAs was included as indicated above the lanes. − 135/ − 52, unlabelled −135 to −52 STH-2 fragment; AP-1, oligonucleotide containing the AP-1 binding sequence [11]; pBS, plasmid pBluescript (Stratagene). I indicates the position of complex I.

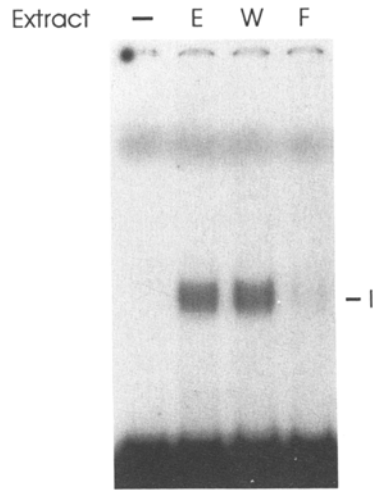

Fig. 7. Binding activity in nuclear extracts from tubers having received different treatments. The probe (STH-2, −135 to −52) was incubated in the absence (−) or the presence of nuclear extracts prepared from elicited (E), wounded (W) and fresh (F) tubers. The same amount of nuclear protein was used in the binding reaction of each extract. I indicates the position of complex I.

complexes using a gel-retardation assay [35]. The ^{32}P-labelled UAS characterized in Fig. 5 was incubated with a nuclear extract from elicited tuber discs. Upon electrophoresis, one retarded band was observed on the gel, indicating the formation of a DNA-protein complex (I) (Fig. 6). Sequence specificity was assessed by including unlabelled specific competitor DNA in the binding reaction. Binding was markedly reduced when a forty-fold molar excess of the −135 to -52 DNA fragment was present, whereas the same molar excess of a heterologous DNA fragment (AP-1 or pBS) had little effect on the formation of the complex. This suggests that the nuclear factor involved in the formation of complex I is specific for the −135 to −52 DNA fragment.

In order to check whether the ability to form complex I was restricted to the elicited tuber preparation, we prepared nuclear extracts from fresh and wounded tubers (tuber discs treated with water instead of arachidonic acid). Figure 7 shows that the intensity of the retarded band is comparable for both the elicited (E) and wounded (W) tuber preparation. In contrast, the nuclear extract prepared from fresh tubers (F) yielded a faint retarded band for complex I. This complex I was also formed when using nuclear extracts from untreated leaves, stems and roots, suggesting that the same factor is present in all parts of the plant (data not shown).

Oligonucleotides covering the region from −134 to −50 were synthesized (see Fig. 8) and

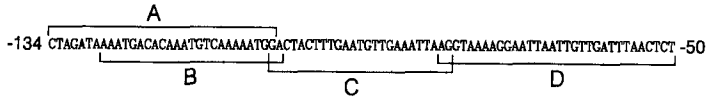

Fig. 8. Position and sequence of the oligonucleotides used in the experiments presented in Fig. 9.

used as probes in the DNA binding assay in order to define more precisely the sequence elements involved in the formation of complex I. No specific binding was observed when oligonucleotides B, C and D were incubated with nuclear extracts from elicitor-treated tubers (data not shown). However, Fig. 9 shows that two retarded bands (complex II and III) are formed with oligonucleotide A. The specificity of the binding to oligonucleotide A was assessed by using a twenty-fold molar excess of each of the four unlabelled oligonucleotides (A, B, C and D) and of the control oligonucleotide AP-1. Only oligonucleotide A could reduce significantly the formation of the DNA-protein complexes II and III. Competition of oligonucleotide A with the −135 to −52 DNA fragment caused a similar reduction of the formation of complexes II and III (data not shown).

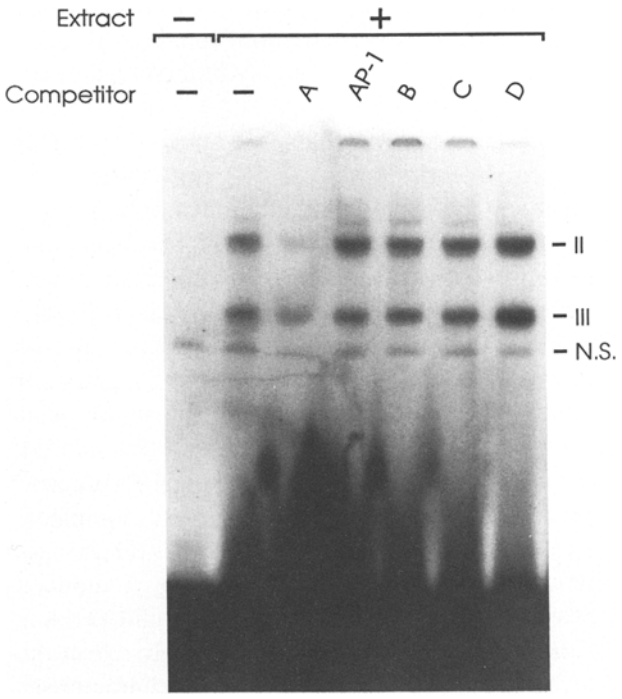

Fig. 9. Binding assays delimiting the DNA sequence responsible for the formation of the complex with the −135 to −52 fragment. Oligonucleotide A was labelled with ^{32}P and incubated in the absence (−) or the presence (+) of the nuclear extract. A twenty-fold molar excess of each specific competitor DNA (oligonucleotides A, B, C, D and AP-1) was included. N.S. indicates a non-specific band. II and III indicate the position of complexes II and III, respectively.

This suggests that the factor(s) involved in the formation of complexes II and III is (are) specific for the oligonucleotide A, which is part of the −135 to −52 DNA fragment.

Discussion

We have shown that two functional copies of the pathogenesis-related genes STH-2 and STH-21 are closely spaced on a single chromosome in potato. The two genes contain a single intervening sequence interrupting the coding region at amino acid 57. The position of this intron is also conserved in pea and parsley homologous genes [5, 40, 44]. The very high similarity that exists between STH-2 and STH-21 suggests that they arose from a recent duplication event [26]. Interestingly, the similarity extends up to approximately 660 bp in the 5′-flanking region and at least 237 bp in the 3′-flanking region, indicating that these non-coding regions were included in the duplicated unit (Fig. 1). The percent identity in these regions is remarkably similar to that observed in the exon regions, and is higher than the percent identity observed between the introns. This suggests that the flanking regions are under some selective pressure preventing them from accumulating mutations as freely as the introns. The high sequence similarity in the 5′ region precluded any attempt to identify distinctive DNA motifs in the two genes.

Analysis of ten transgenic plants transformed with a Ti-plasmid containing the STH-2 −1015 to +145 bp region fused to the GUS reporter gene indicated that this region contains *cis*-acting elements necessary for the wound and elicitor-induction of the gene. The levels of activity observed in these transgenic plants parallel those observed for the accumulation of STH-2 mRNAs under similar conditions. In the transgenic plants, the ratio of GUS activity in elicitor-treated over wounded tubers is approximately 13-fold, while the ratio of STH-2 mRNAs in the same tissues is about 10-fold [29]. Similarly, the absence of a significant level of GUS activity in unsliced minitubers is in agreement with the lack of accumulation of STH-2 mRNAs in these tissues [29].

This further indicates that the transgene is normally regulated and under tight control in the transgenic plants.

Results from deletion analysis of the STH-2 gene 5′-flanking region suggested that 135 bp of upstream region may be sufficient for full promoter activity in the transient expression system. Our results further indicated the presence of an important regulatory sequence localized between nucleotides −135 and −105 (Fig. 4). This sequence showed some properties characteristic of a promoter enhancer: it worked independently of its orientation when positioned upstream of the STH-2 TATA element, its duplication had an additive effect on reporter gene activity (Fig. 5) and it also increased the activity of an heterologous promoter (CaMV 35S minimal promoter). However, the activity was decreased when the element was positioned at a distance, and no enhancing effect could be detected when the element was inserted downstream of the TATA box of the CaMV 35S minimal promoter (not shown). The reduced expression observed with the −670Δ-UAS gene construction, which is identical to the −670 deletion except for an internal deletion of the −135 to −52 region (compare Fig. 5G and 5H), confirmed that this element is necessary for full promoter activity in the transient expression system. Our results, however, do not eliminate the possibility that the region upstream of −135 contains other regulatory elements necessary for gene regulation *in planta*. Furthermore, the high basal expression level that remains in the −27 deletion and the loss of this activity in the −5 deletion indicate that a regulatory element is present in the −27 to −5 region. Although this element could be the TATA box which is present at positions −25 to −18, it is also possible that another element is present further downstream, and that this element requires the presence of a functional TATA box at position −25 to exert its action.

The functional significance of the −135 to −52 region for the expression of the STH-2 gene was strengthened by the observation that this region interacts with at least one specific nuclear protein. Furthermore, oligonucleotide A, which corresponds almost exactly to the sequence which is necessary for full promoter activity in transient assays, gave rise to the formation of two DNA-protein complexes. The fact that the oligonucleotide B, which overlaps with oligonucleotide A except for the first 7 nucleotides, does not lead to the formation of a retarded complex suggests that this 7 bp region plays an important role in the establishment of the DNA-protein complex. Interestingly, a 14 bp imperfect palindromic sequence, −124 TGACACAAATGTCA which resembles the TGAC DNA-binding motif found in many promoters [21, 38, 42], is immediately adjacent to these nucleotides. A TGAC motif is also present in the sequence of the parsley PR1-1 gene, whose product shares 37% amino acid identity with the STH-2 protein. In the PR1-1 promoter, this sequence (−240 TGACCGAGTAA) is part of a region which is protected *in vitro* from methylation by dimethyl sulphate [32]. This further suggests that the TGAC motif found at position −124 in the STH-2 gene may be involved in the regulation of the gene.

The observation that the nuclear factor is present in most parts of the plant (tuber, root, stem and leaf) suggests that it may not be directly involved in the induction of the STH-2 gene following wounding or elicitor treatment, but may nevertheless be required for the induction to take place. The factor could play a role through an interaction with another, inducible, factor or could require activation through some post-translational modifications. The fact that a larger quantity of complex I is formed when the nuclear proteins are isolated from wounded or elicitor-treated tubers rather than from fresh tubers suggests that the synthesis of the factor is induced after wounding or that its ability to bind DNA is enhanced. However, this could simply reflect the low transcriptional activity which characterises unwounded potato tubers. Results presented in Fig. 7 also suggest that, unless it requires *trans*-activation, this factor is not directly involved in the increased expression of the gene following elicitor treatment, since the abundance of complex I remains the same after wounding or elicitor treatment.

The presence of a negative regulatory element in the promoter of STH-2 was first suggested by the small but reproducible increase in activity of the reporter gene observed when the −52 to −27 region was deleted (Fig. 4). This result was supported by the five-fold increase in GUS activity obtained following the internal deletion of nucleotides −51 to −28 (Fig. 5F). It is therefore possible that this region negatively regulates the expression of the gene by binding to a specific factor. On the other hand, the increased activity could be the result of the change in the spacing between the UAS and the TATA box at position −25, favouring a better interaction between a UAS-binding transcriptional factor and RNA polymerase II or other general transcriptional factors binding near the TATA box.

Addition of elicitors before or after preparation of the protoplasts and/of electroporation had no effect on the activity of the reporter gene (results not shown). We speculate that the protoplast isolation procedure was sufficient in itself for induction of the expression of wound- and elicitor-responsive genes. There are many reported examples showing that this might be the case. For example, Jouanneau *et al.* [20] showed that defence-related genes were highly expressed in freshly isolated protoplasts of soybean and the pea DRRG49 gene, which shares high sequence identity with STH-2, was also active in unelicited electroporated tobacco protoplasts [5].

An interesting comparison can be made between our results and those obtained by van de Löcht *et al.* [44] with the elicitor-induced parsley PcPR2 gene, whose product shares 39% amino acid identity with the STH-2 protein. In a transient expression system with parsley protoplasts, a 125 bp promoter fragment of the PcPR2 gene was demonstrated to be sufficient for elicitor-mediated gene activation. This element is localized between nucleotides −168 and −52, and regions upstream of −168 were not necessary for reporter gene activity. This is similar to our results which show that the region upstream of −135 is not necessary for reporter gene activity in electroporated protoplasts. Comparison of these regions in both genes revealed a short stretch of high similarity where 13 out of 14 nucleotides were identical (−134 TGAA*G*TTGAAATT for PcPR2 and −96 TGAA*T*GTTGAAATT for STH-2). Part of this region (GTTGAAATT) is also found in the 5′-flanking region of the wound and pathogen-inducible potato *wun1* and *win1* genes [37, 41].

In conclusion, our results indicate that a small region of the potato STH-2 PR gene is necessary for full promoter activity in electroporated protoplasts, and that a nuclear factor is able to bind to this region. Transgenic plants harboring different deletions and chimaerae will determine if this short region is also important *in planta* and if other regulatory regions necessary for tissue-specific expression and developmentally controlled expression are present in the STH-2 promoter.

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