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Key words: Arabidopsis thaliana, Brassica oleracea, plant defence, WRKY

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Abbreviations: as-1, activation sequence-1; *ARK1/2/3,* Arabidopsis receptor kinase 1/2/3; BTH, benzothiadiazole; CaMV, cauliflower mosaic virus; Col-0, Columbia-0; GUS, β-glucuronidase; *gusA*, β-glucuronidase gene; HR, hypersensitive response; INA, 2,6 dichloroisonic acid; ISR,

induced systemic resistance; JA, jasmonic acid; KCl, potassium chloride; 4-MUG, 4-methylumbelliferyl-D-glucuronide; 4-MU, 4-methylumbelliferyl; *NahG*, salicylic hydroxylase; PR, pathogenesis related; PRK, plant receptor kinase; *RLK1*, receptor-like kinase 1; *RKS1*, receptor kinase of the S domain class 1; SA, salicylic acid; SAR, systemic acquired resistance; *SFR1/2/3*, S family receptor 1/2/3, SLG, S locus glycoprotein; SRK, S locus receptor kinase; WAK1, wall associated kinase 1

Abstract

Transcripts of *SFR2*, a member of the *S* family of receptor kinase genes, accumulate rapidly in *Brassica oleracea* leaves in response to wounding, bacterial infection and following treatment with salicylic acid (SA). Expression of a chimeric gene consisting of the *SFR2* 5' flanking sequence fused to the *gusA* reporter gene is also induced in wounded and SA-treated *Arabidopsis* plants indicating that the observed response is conferred by the *SFR2* promoter. We show here that, in *Arabidopsis* plants carrying the salicylate hydroxylase (*NahG*) transgene, wound induction of the *SFR2* promoter-*gusA* reporter fusion was abolished, indicating that, as has previously been shown for the response to bacterial infection, SA is required for the response to wounding. Deletion analysis of the *SFR2* promoter identified a region necessary for full expression following SA treatment. This region, which includes two putative W-boxes, is conserved in the promoter of the *Arabidopsis SFR2* homologue, *ARK3*. Deletion of a 12 bp region containing the two W-box motifs reduced the response to SA treatment. Tandem repeats of the W-box-containing element fused upstream of a CaMV *35S* minimal promoter enhanced reporter gene expression in transgenic *Arabidopsis* both in the absence and presence of SA. Gel-mobility shift assays showed that *Arabidopsis* leaf extracts contained factors that bound to a fragment of the promoter spanning the putative W-boxes and that a fragment in which these motifs were mutated was unable to compete for binding. In summary, induction of the *SFR2* promoter in response to bacterial infection and wounding requires SA and full expression of the induced gene requires the presence of a functional element containing W-box motifs in the *SFR2* promoter. The involvement of two W-boxes indicates that transcription factors of the WRKY family may play a key role in mediating these responses.

1. Introduction

Salicylic acid (SA), a product of plant secondary metabolism derived from the phenylalanine ammonium lyase and isochorismate synthase pathways, plays a key role in plant defence responses to pathogens (Vernooij et al., 1994). When invaded by pathogens, resistant plants induce defence mechanisms both locally and in distant, non-infected tissues. Local defences are usually associated with a rapid, programmed cell death at the site of infection termed the hypersensitive response (HR). The various molecular responses accompanying HR restrict growth of the pathogen and its spread throughout the plant. Exposure to pathogens that cause necrosis can also induce systemic acquired resistance (SAR), which confers sustained immunity to further infection by a broad spectrum of pathogens (Hunt et al., 1996). In many plant-pathogen interactions, HR and SAR are accompanied by the accumulation of a large group of pathogenesis related (PR) proteins.

In both *Arabidopsis* and tobacco, SA is generally necessary and sufficient for the induction of HR and SAR. Application of exogenous SA triggers synthesis of PR proteins and confers resistance to pathogens (Yang et al., 1997). These same two phenomena, PR protein production and induced resistance, were observed in transgenic tobacco plants that produced SA constitutively (Verberne et al. 2000). Increases in the levels of endogenous SA and its conjugates in pathogen infected wild type plants correlate with the expression of PR genes and the development of disease resistance. Furthermore, HR, PR gene expression and disease resistance are compromised in *Arabidopsis* plants carrying mutations that prevent SA accumulation such as *eds1* and *pad4* (Shah, 2003). Similar observations have been made with transgenic tobacco and *Arabidopsis* plants, where SA accumulation is prevented by the constitutive expression of the

NahG transgene (Gaffney et al. 1993). *NahG* encodes a bacterial salicylate hydroxylase which converts SA to biologically inactive catechol, but does not metabolise two synthetic functional analogs of SA, 2,6 dichloroisonicotinic acid (INA) and benzothiadiazole (BTH). These compounds can restore disease resistance in SA-depleted *NahG* tobacco and *Arabidopsis* plants. Although these different lines of evidence argue for an essential role for SA in the onset of SAR, graft experiments demonstrate that SA is neither the translocated signal nor is it required for generation of the translocated signal. However, SA is required to mediate the translocated signal, the nature of which remains unknown (reviewed in Vernooij et al., 1994).

Transmembrane sensors play an essential role in perceiving external stimuli from the environment as well as stimuli communicated through the plant body by endogenous signals. In *Arabidopsis*, plant receptor kinases (PRKs; Cock et al., 2002) constitute the largest recognizable class of transmembrane sensors; a superfamily of 417 PRK genes has been described in this species (Shiu and Bleeker, 2001). The PRK superfamily is monophyletic with respect to the kinase domain but can be subdivided into at least 21 classes based on the sequence of the putative extracellular domain. Plant PRKs play important roles in various development processes and in defence responses to both biotic (e.g. disease) and abiotic stresses (e.g. wounding). Thus, the polypeptide systemin which mediates systemic wound signalling in tomato binds to the PRK SR160 (Scheer and Ryan, 2002), *Arabidopsis* FLS2 is involved in pathogen recognition mechanisms via perception of flagellin, a bacterial elicitor, and subsequent phosphorylation of intracellular target proteins (Gomez-Gomez and Boller 2000) and, in monocots, the rice PRK Xa21 mediates resistance to the bacterial pathogen *Xanthomonas oryzae oryzae* (Song et al., 1995). The fact that expression of several PRKs has been shown to be induced following SA treatment also argues that these molecules play a general role in the defence response (Ohtake et al. 2000; Du and Chen, 2000; Pastuglia et al., 1997, Pastuglia et al., 2002).

The first PRK gene shown to be induced during the defence response was *S gene family receptor 2* (*SFR2*) from *Brassica oleracea* (Pastuglia et al., 1997). The *SFR2* gene was isolated in a screen for vegetatively expressed genes related to the self-incompatibility locus genes *S locus glycoprotein* (*SLG*) and *S locus receptor kinase* (*SRK*; Stein et al., 1991). Additional, expressed members of the S domain class of PRK genes have been described in *Brassica oleracea* (*SFR1* and *SFR3*; Pastuglia et al., 2002) and *Arabidopsis* (e.g. *RLK1, RKS1, ARK1, ARK2* and *ARK3*; RLK for receptor-like kinase, RKS for receptor kinase of the S domain class, *ARK* for *Arabidopsis* receptor kinase; Tobias et al. 1992; Dwyer et al. 1994; Walker, 1993; Ohtake et al., 2000).

In contrast to *SRK*, *SFR2* is expressed in vegetative tissues, mainly in leaves. *SFR2* was shown to be rapidly induced in leaves of *Brassica oleracea* by wounding, bacterial infection and salicylic acid, indicating a role in the defence response. Experiments in transgenic tobacco have demonstrated that the 1516 bp upstream of the *SFR2* coding region is sufficient to induce expression of a fused *gusA* reporter gene in response to SA treatment and bacterial infiltration (Pastuglia et al., 1997). Similarly, the 1516 bp promoter of SFR2 is activated upon bacterial infection in *Arabidopsis* (Pastuglia et al., 2002).

Here we show that induction of *SFR2* by wounding requires SA, as has been previously observed for the induction of this gene following bacterial infection. Analysis of the *SFR2* promoter identified a *cis*-acting element that plays a major role in mediating the SA response.

2. Materials and methods

2.1 Plant material and growth conditions

The *Arabidopsis thaliana* ecotype used was Columbia-0 (Col-0). Seed from transgenic *Arabidopsis* Col-0 plants expressing the *NahG* gene under the control of the enhanced CaMV 35S promoter from CaMV was obtained from John Ryals.

Plants were grown on soil in a growth chamber with a 12 hrs light/dark period at 20°C (light) / 14°C (dark). Treatments were performed after 8 to 10 weeks growth (prior to bolting).

2.2 Promoter constructs

Cloning of the 1516 bp *SFR2* promoter region upstream of the *gusA* reporter gene has been described by Pastuglia et al. (1997). Deletions from the 5' end of the 1516 bp *SFR2* promoter fragment (Fig. 2) were generated by PCR using specific primers with 5' extensions containing HindIII and XbaI restriction sites and cloned into pBI101.

Internal deletions within the 1516 bp *SFR2* promoter fragment, 1516Δ57 (-504/-448) and 1516Δ12 (-494/-483), were obtained by a gene splicing approach referred to as splicing by overlap extension and cloned into pBI101. Briefly, DNA fragments on either side of the deletion were amplified in separate PCR reactions. Primers on either side of the deletion contained 5' end extensions so that they were complementary over 18 nucleotides. When the two primary PCR products were mixed in a second round of PCR, the strands having the matching sequences at their 3' ends acted as primers for each other and extension of this overlap by DNA polymerase produced the promoter fragment with the desired deletion. This secondary PCR product was amplified by external primers with 5' extensions containing HindIII and XbaI restriction sites for cloning in pBI101.

Synthetic promoters containing tetramers of putative regulatory elements and a minimal

promoter sequence were constructed as follows. The cauliflower mosaic virus (CaMV) 35S minimal promoter (-46/+8) plus the 5' end of the *gusA* gene was amplified by PCR from the pBI121 vector (Clontech) creating a SpeI restriction site at its 5' end, subcloned in pGEM-T Easy (Promega, Charbonnières, France) and excised with SalI and SnaBI restriction enzymes for cloning into pBI101. After digestion of the recombinant plasmid with HindIII and SpeI, tandem repeats of putative regulatory elements were cloned immediately upstream of the CaMV 35S minimal promoter.

To obtain tandem constructs of putative regulatory elements, PCR fragments flanked by SpeI and XbaI restriction sites were ligated into pGEM-T Easy (Promega), excised with PstI and XbaI, and transferred repeatedly to pBCSK+ (Stratagene, La Jolla, USA) to obtain clones containing four tandem copies in the sense orientation. Tetramers were excised with HindIII and XbaI and fused upstream of the CaMV 35S minimal promoter in pBI101. A tetrameric repeat of the *SFR2* promoter *cis* element (bases -501 to -473, Fig. 4B) was designated 4xWT. The TGAC core of the putative W-box in the 4xWT element was modified to TCTT to obtain a mutated version designated 4xMUT (Fig.4B). A tetrameric repeat of a region containing the as-1 element (region -93 to -60 of the CaMV 35S promoter), designated 4xAS1, was obtained using the same approach. The *as-1* element is located at bases -83 to -63 in the CaMV 35S promoter.

Two DNA fragments directly upstream of *ARK3* coding sequence, of 1428 bp and 429 bp, were amplified by PCR. The PCR products were cloned into pBI101 using HindIII and XbaI restriction sites at their extremities.

All promoter constructs were verified by DNA sequencing.

2.3 Arabidopsis *transformation and crosses*

All constructs were assembled in the binary vector pBI101 (Clontech) and introduced into *Arabidopsis* Col-0 by vacuum infiltration (Bechtold et al., 1993) via *Agrobacterium* strain EHA105. Selection of transgenic plants was carried out on MS plates (Murashige and Skoog, 1962) containing 50mg/l kanamycin sulfate.

For *SFR2p::gusA* constructs, lines carrying single T-DNA insertions were selected after selfing of the primary transformants (T_1) and T_2 plants homozygous for the transgene were identified by analysis of T_3 progeny. T_2 plants carrying T-DNA insertions at single loci, as determined by segregation of kanamycin resistance, were used for SA treatment.

T_3 plants homozygous for the 1516 bp *SFR2p::gusA* transgene were crossed with *Arabidopsis* plants homozygous for the *NahG* transgene to obtain F_1 populations carrying the two transgenes for wounding experiments. F_2 progeny of this cross were analysed to verify that the *SFR2* gene fusion had not become non-functional.

2.4 SA treatment and wounding of Arabidopsis *leaves*

For the wounding experiments, *Arabidopsis* Col-0 carrying one copy of the 1516 bp *SFR2p::gusA* transgene plus the *NahG* transgene, and plants carrying the 1516 bp *SFR2p::gusA* transgene alone were used. Control leaves were harvested before wounding, which was performed by piercing repeatedly (approx. 20 times) one middle-size leaf on each plant with a 0.3 mm needle. Wounded and systemic leaves were collected 1 and 7 days after wounding, frozen in liquid nitrogen, and stored at –80°C. Twenty five plants (pooled) from each of 4 F_1 families obtained from 4 independent crosses were analysed. Control experiments were carried out on F_2 progeny plants.

SA treatments were performed using two different protocols. Firstly, an assay using detached leaves was carried out on primary transformants for each of the *SFR2* promoter 5'

deletion constructs. Three leaves were removed from each plant and each was subjected to a different treatment: incubation for 1 hr in 1 mM SA (sodium salt; Sigma, Lezennes, France) in 50 mM sodium phosphate buffer, pH 7, incubation for 1 hr in phosphate buffer alone or untreated. Leaves were then rinsed three times in phosphate buffer before incubation for 20 h in liquid MS media at 20°C under continuous light. Samples were frozen in liquid nitrogen and stored at -80°C. The same protocol was used to test tandem repeat constructs.

Secondly, the effect of spraying with SA was tested for each of the ten 5' deletion constructs using 25 plants each from 15-20 independent transgenic T_2 families carrying single transgene insertion loci per construct. Rosette leaves were harvested 24 hrs after spraying with 1 mM SA in 50 mM sodium phosphate buffer, pH 7 or with phosphate buffer alone. Internal deletion constructs were tested in the same way using 3 to 4 pools of 8 T_1 plants originating from independent transformation events for each constuct.

2.5 Fluorometric GUS assays

Extracts were prepared in GUS assay buffer and reactions carried out essentially as described by Jefferson et al. (1987) using 4-methylumbelliferyl-D-glucuronide (4-MUG, Sigma) as a substrate. The concentration of the fluorescent product, 4-methylumbelliferyl (4-MU), was determined with a Fluoroskan II Elisa plate reader. The amount of protein in each extract was measured in a Bradford assay (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instruction.

2.6 EMSA

Soluble whole cell protein extracts were prepared essentially as described by Foster et al. (1992). Probes and competitors were generated by excision with NotI of 4xWT, 4xMUT and 4xAS1 inserts from recombinant pBCSK+ plasmids (see "Promoter constructs" above, Fig.5). After purification from an agarose gel, the 4xWT fragment was end-labelled with the Klenow fragment of DNA polymerase I and [-^{32}P]dGTP (3000 Ci/mmol, NEN, Boston, USA). The probe was purified by electrophoresis through a 6% polyacrylamide gel, followed by an overnight elution in water at 4°C, filtration and ethanol precipitation.

Binding reactions were performed for 30 min at room temperature with 6 ng of labeled DNA probe (15,000 cpm), 2 g poly[dIdC]/ [dIdC], and 7.5 g of total protein extract in a final volume of 10 µl binding buffer containing 15 mM Hepes pH 7.5, 35 mM KCl, 0.4 mM EDTA and 6% glycerol. The reactions were loaded on a 6% non-denaturing polyacrylamide gel (29% acrylamide, 1% bis-acrylamide) in 0.5 x TBE (Tris-borate 45 mM, EDTA 1mM) after a pre-run for 1 h at 150 V. Electrophoresis was then carried out overnight at 80 V. The gel was fixed in 10 % acetic acid for 10 min, dried at 80°C under vacuum for 2 hrs and autoradiographed using Hyperfilm (Amersham, Orsay, France). Competition experiments were carried out by adding unlabelled competitors (4xWT, 4xMUT and 4xAS1) to the binding reaction at molar ratios of 30 or 300 compared to the probe.

3. Results

3.1 Induction of SFR2 *following pathogen infection and wounding requires an SA signal*

SFR2 mRNA accumulates in response to wounding but no accumulation is detected following

treatment with methyl jasmonate (Pastuglia et al., 1997), a molecule that has been implicated in signal transduction pathways leading to the induction of many wound-inducible genes (Ryan and Moura, 2002). We considered the possibility that wound-induced expression of *SFR2* could be mediated by SA based on two observations: Firstly, the accumulation of *SFR2* transcripts in response to a bacterial pathogen requires SA (Pastuglia et al., 2002) and, secondly, the kinetics of accumulation of *SFR2* mRNA in response to wounding is similar to that to that observed following SA treatment (Pastuglia et al., 1997). To test the possible role of SA in wound induction, a gene fusion containing the *gusA* reporter gene under the control of a 1516 bp promoter, corresponding to the region immediately upstream of the *SFR2* coding sequence, was stably transformed into *Arabidopsis.* Plants homozygous for the transgene were crossed with plants that had been rendered incapable of accumulating SA due to expression of a salicylic acid hydroxylase encoded by the *NahG* gene (Gaffney et al., 1993). Leaves of F_1 plants originating from four independent crosses were wounded by repeatedly piercing with a needle. As a control, the same treatment was performed on a line that was homozygous for the *SFR2p::gusA* fusion but without the *NahG* gene. Figure 1 shows that, in the absence of *NahG*, accumulation of β-glucuronidase activity was detected in both the wounded leaves themselves and in adjacent leaves. One day after wounding, the level of β-glucuronidase in wounded as well as adjacent leaves had increased but was not significantly different from that of unwounded leaves (note, however, that, because whole leaves were extracted, a very localised induction, in the punctured regions of the leaves, may not have resulted in a significant induction of gene expression). A stronger (and statistically significant) systemic induction of the transgene was however observed seven days after wounding. In contrast, in the presence of *NahG*, the basal level of ß-glucuronidase activity was significantly lower than that detected in plants without the *NahG* gene

and the *SFR2p::gusA* transgene was not induced by wounding. To verify that the low β-glucuronidase activities measured in *NahG* plants were not due to loss or inactivation of the *SFR2p::gusA* transgene after crossing, we screened progeny of the F_1 hybrid plants by PCR to identify F_2 plants carrying the *SFR2p::gusA* transgene but not *NahG*. Following wounding, the levels of β-glucuronidase activity in these plants were similar to those previously observed for plants homozygous for the *SFR2p::gusA* fusion (data not shown). In conclusion, these data, together with those presented by Pastuglia et al. (2002), indicate that *SFR2* is induced systemically in response to wounding and that SA is required both for the basal level of expression of *SFR2* and for its induction following wounding and pathogen infection.

3.2 Deletion analysis of the SFR2 *promoter region*

Transcripts of the *SFR2* gene have been shown to accumulate rapidly and transiently in *Brassica* leaves following treatment with 1 mM SA and a 1516 bp fragment directly upstream of the *SFR2* coding sequence conferred SA inducibility when incorporated into a *gusA* reporter gene fusion (Pastuglia et al., 1997). To identify regulatory elements within this promoter fragment, we created a series of ten 5' deletions of the *SFR2* promoter, fused to the *gusA* gene (Fig. 2A). The effect of SA on the transcriptional activity of these constructs was then assayed using two different approaches in transgenic *Arabidopsis* plants.

Firstly, leaves were detached from 25 to 40 T_1 generation plants for each deletion construct and immersed in either phosphate buffer containing 5 mM SA or phosphate buffer alone or were harvested without treatment. This approach allowed rapid comparison of the different transgene constructs. Figure 2B shows that treatment with SA resulted in an increase in GUS activity,

compared with the phosphate buffer controls, for most constructs. GUS activity in phosphate buffer-treated leaves of primary transformants was higher than that of untreated controls, probably due to the slight wounding resulting from leaf removal. SA-inducibility was conferred by a 504 bp fragment of the promoter but was lost when this fragment was further deleted to 343 bp.

To provide a more precise measurement of transgene activity, analyses for each construct were carried out on T_2 plants from 15-20 lines with single T-DNA insertion loci, each line representing an independent transformation event. These plants were sprayed with either 1 mM SA or with phosphate buffer as a control. A high level of variability was observed between the levels of activity for different lines carrying the same construct, as previously reported for the *PR1* gene (Lebel et al., 1998). Therefore, a large number of lines were analysed for each construct to minimize variability among lines. As a result of this variability, however, comparison of average fold induction was more informative than comparison of average values for GUS activity (see Fig. 2).

With the exception of the 612 bp promoter fragment, spraying with SA gave similar results to immersion of detached leaves (Fig. 2C). Spraying with SA resulted, on average, in a three-fold induction of GUS activity using the 504 bp promoter fragment, whilst there was a complete loss of inducibility by SA with shorter promoter fragments (Fig. 2C). Note also that deletion of sequences upstream of –740 resulted in a loss of induction by SA but that inducibility was restored when further sequence was deleted (Fig. 2C). A similar phenomenon was observed in the experiment using primary transformants (Fig. 2B). These data indicated the presence of additional regulatory elements in the upstream region of the promoter. However, we decided to focus on the region of the *SFR2* promoter between positions -504 and –447 because the deletion experiments indicated that this region was clearly necessary for SA-inducibility.

3.3 Identification of a conserved sequence in the Brassica SFR2 *and the* Arabidopsis ARK3 *promoters*

The *Arabidopsis* genome is predicted to encode a total of 36 S gene family receptor kinases (S. Riviere and JMC, unpublished data). The *Brassica* SFR1, SFR2 and SFR3 receptor kinases are most similar to the *Arabidopsis* ARK1, ARK3 and ARK2 receptor kinases, respectively (Pastuglia et al., 2002). SFR2 shares 81.5% identity with ARK3 and a neighbour joining tree constructed from an alignment of the amino acid sequences of the SFR and ARK proteins supported the close relationship between these two proteins (Fig. 2A).

ARK3 mRNA has been shown to accumulate in response to both wounding and infection by a bacterial pathogen (Pastuglia et al., 2002). To test whether *ARK3* transcription is also induced in the presence of SA, we fused 1428 bp of the *ARK3* promoter to the *gusA* reporter gene and transformed this construct into *Arabidopsis*. Rosette leaves of primary transformants were sprayed with 1mM SA, harvested and assayed for β-glucuronidase activity. Figure 3B shows that induction of GUS expression driven by 1428 bp of the *ARK3* promoter region was three fold stronger following addition of exogenous SA than when the plants were sprayed with phosphate buffer alone.

The similarity of the responses of *SFR2* and *ARK3* to wounding, bacterial infection and exogenous SA prompted us to compare the promoter regions of these two genes with the aim of identifying shared sequence motifs. Dot-matrix comparison of the *SFR2* and *ARK3* promoters showed that they shared no overall sequence similarity (data not shown) but a short region showing significant similarity was identified (Fig. 3C). In the *SFR2* promoter, this sequence is

located within the region (-504 to -447) that we had earlier shown to be required for induction by SA (Fig. 2). This suggested that a conserved element within this region may be important in mediating the effect of SA on the transcriptional activity of the *SFR2* and *ARK3* promoters. The response of a 5'-truncated *ARK3* promoter of 429 bp (and, therefore, still containing the conserved fragment) fused to *gusA* to SA treatment supported this hypothesis (Fig. 3B).

The conserved region is part of a 107 bp imperfect repeat in the *SFR2* promoter and a second copy of the conserved element occurs further downstream (Fig. 3C). The role of the downstream copy of this region is unclear, however, as constructs that contained this region alone (eg. the 447 and 402 bp constructs in Fig. 2A) did not confer SA inducibility to the chimeric reporter gene. Moreover, the conserved region is not repeated in the *ARK3* promoter indicating that duplication of the region is not necessary for its function.

The conserved region shared by the *SFR2* and *ARK3* promoters includes a pair of tandemly repeated TGAC sequences in *SFR2* (Fig. 3C). The TGAC motif is found as an invariant core sequence in the so-called W-boxes, first identified as *cis* elements which bind transcription factors of the WRKY family and mediate a transcriptional response to pathogen-derived elicitors (Eulgem et al., 2000). Two types of W-box have been described, with the concensus sequences (T)(T)TGAC(C/T) and TGAC-$(X)_n$-GTCA (Eulgem et al., 2000). W-boxes were first identified as *cis* elements mediating a transcriptional response to pathogen-derived elicitors via binding to transcription factors of the WRKY family (Eulgem et al., 2000). W-box consensus sequences have been found in the promoters of a number of SA-inducible genes and have been shown to be necessary for induction by SA in some cases (Du and Chen, 2000, Ohtake et al., 2000).

The TGAC motif also occurs in activation sequence 1 (as-1), a *cis*-acting element that is comprised of a tandem repeat of the sequence TGACG (Lam et al., 1989; Fig. 5A). The as-1 element has been reported to confer SA-inducibility on a 90 bp fragment of the CaMV 35S

promoter (Qin et al., 1994). SA-induced activation has been correlated with the binding of bZIP transcription factors of the TGA family to this element (Jupin and Chua, 1996). Note, however, that the motif identified in the *SFR2* and *ARK3* promoters more closely resembles the W-box consensus than it does as-1.

The members of the *SFR* and *ARK* gene families show different patterns of expression and respond differently to stimuli such as bacterial infection and wounding (Fig. 3A). To extend the comparison to other members of these families, we cloned the upstream regions of *SFR1* and *SFR3* using a genome PCR-walking protocol (EMBL accession numbers **AJ495783** and **AJ495782**, respectively) and retrieved the *ARK1* and *ARK3* upstream regions from the *Arabidopsis* genome database. Dot-matrix comparison of the *SFR2* promoter region with the *ARK1* (1.6 kbp), *SFR1* (2 kbp) and *SFR3* (1 kbp) 5' flanking regions did not identify any conserved sequence of more than 9 bp.

3.4 Functional analysis of the conserved region of the SFR2 *promoter*

To investigate further the role of the conserved region of the *SFR2* promoter in the response to SA, we deleted either a 57 bp region (-504 to -448) corresponding to the sequence required for SA induction that was identified by deletion analysis, or part of this sequence, namely a 12 bp region that included the putative W-box (-494 to -483, GGTTGACTGACT, where the putative W-box is underlined). Promoters with these internal deletions were fused to the *gusA* gene and transformed into *Arabidopsis*. Levels of β-glucuronidase activity 24 hrs after treatment with 1 mM SA were significantly lower in transgenic plants carrying the two deletion constructs than in the undeleted control (Fig. 4A), the 57 bp deletion having the most marked effect. However,

neither deletion entirely eliminated the response to SA and, although absolute levels of GUS activity were significantly reduced, inducibility following SA treatment was highly variable and the reduction in SA inducibility was not statistically significant. This high level of variability was observed despite the fact that a considerable effort was made to reduce sampling errors (assays were conducted on pools of 25 plants each from 15-20 independent transgenic T_2 families for each construct) suggesting that the effect of the deleted regions on SA inducibility was strongly influenced by the chromosomal contexts of the inserted transgenes. Because of this variability, these constructs did not allow us to confirm that the conserved region is necessary for SA induction. Nonetheless, these data clearly indicate that the sequence between positions -494 and -483 is necessary for full activity of the *SFR2* 1516 bp promoter following induction by SA-treatment, indicating that the conserved region acts, at least in part, as an enhancer. Enhancer effects of W-box sequences have been described in other studies (eg. Du and Chen, 2000).

Involvement of adjacent regions cannot be excluded however as deletion of the –494 to –483 fragment may have consequences on binding of trans-acting elements to these regions due to altered spacing and/or DNA bending.

To investigate further the role of the conserved region in *SFR2* promoter regulation, we constructed a synthetic promoter element, designated 4xWT, which consisted of four copies of a 29 bp region from -501 to -473. The 4xWT element was placed upstream of a minimal CaMV *35S* promoter in front of the *gusA* reporter gene (4xWT-46, Fig. 4B). Fusion of the 4xWT sequence to the minimal promoter resulted in a significant increase in expression levels under all conditions examined. However, inducibility following SA treatment (compared with the phosphate buffer control) was not increased compared to a control construct which contained the -46 promoter alone. These data, which indicate that the conserved region acts essentially as an enhancer, are consistent with those obtained when the region was deleted (Fig. 4A). A similar

result was obtained with a construct containing four tandem copies of the CaMV 35S promoter as-1 element (Fig. 4B). A construct carrying the *SFR2* sequence with the tandem TGAC sequences mutated (4xMUT), did not confer higher levels of expression to the minimal promoter indicating that the increased transcriptional activity associated with the 4xWT-46 construct is correlated with the presence of a functional W-box motif.

3.5 The conserved region of the SFR2 *promoter interacts with cellular factors* in vitro

The ability of the 29 bp region of the *SFR2* promoter surrounding the putative W-boxes (-501 to –473) to bind to cellular factors was tested in electrophoretic mobility shift assays (EMSAs). Figure 5 shows that four major shifted bands were detected after incubation of the 4xWT probe with total soluble protein extracts from *Arabidopsis* rosette leaves. These bands were detected using extracts both from untreated plants and from plants that had been sprayed with 1 mM SA, suggesting that the factors that bind to this part of the *SFR2* promoter are present constitutively within the cell. However, it is possible that either the availability or the activity of binding factors is modified in the presence of SA. For example, since total cellular extracts were used for these experiments, we would not have detected movement of factors between the cytoplasm and the nucleus.

To evaluate the specificity of the DNA-protein complexes, competition experiments were conducted by adding increasing amount of specific competitors. Figure 5 shows that binding was strongly competed in the presence of the wild type sequence (4xWT) but not in the presence of the mutant competitor in which the W-box motif had been altered (4xMUT). Competition was clearly observed at molar ratios as low as 10 for the wild type sequence (data not shown) but could not be detected at a molar ratio of 500 for the mutated sequence.

Part of the W-box motif (TGAC) is very closely related to the two tandem repeats found in the as-1 element from the CaMV *35S* promoter (TGACG). Both types of *cis*-acting element have been shown to mediate SA-inducibility. However, the signalling pathways involved are probably distinct since W-boxes interact with zinc finger transcription factors of the WRKY superfamily whilst the as-1 element binds bZIP transcription factors of the TGA family. To investigate which of these two pathways might be involved in the activation via the *SFR2* element, we tested the ability of the detected DNA-binding activities to interact with the as-1 element from the CaMV *35S* promoter. As shown on Fig. 5, the as-1 element competed poorly with the 4xWT probe even at a molar excess of 300, indicating that the protein(s) involved have a low affinity for as-1, despite the sequence similarity.

4. Discussion

The data presented here, taken together with previous studies (Pastuglia et al., 1997, Pastuglia et al., 2002), demonstrate that the transcriptionally regulated accumulation of *SFR2* mRNA following both wounding and bacterial infection requires the presence of SA, indicating that the signal transduction pathways downstream of these stimuli converge on SA.

The requirement of SA for wound-induced accumulation of *SFR2* mRNA is surprising as this molecule is not normally considered to be a mediator of wound-induced signalling. SA does not accumulate in response to wounding in tobacco (Malamy et al., 1990; Sano et al., 1994) and SA has even been shown to act as an inhibitor of jasmonic acid (JA) signalling in tomato (Doares et al., 1995). However, although SA- and JA-dependent pathways are generally considered to be antagonistic, there is evidence that they can act synergistically under some conditions. Microarray analysis performed on *Arabidopsis* plants showed a large overlap of genes induced or repressed

following JA and SA treatments (Schenk et al., 2000). An additive effect on the level of protection against pathogens has been observed following simultaneous induction of SA-dependent SAR and JA-dependent ISR (Induced Systemic Resistance, van Wees et al., 2000). Moreover, Xu et al. (1994) showed that treatment of tobacco seedlings with JA and SA led to a greater level of induction of PR1 than treatment with either of these molecules alone. These observations may also be directly relevant to the wound response as convergence of pathogen- and wound-response signal transduction pathways at the level of a MAP kinase (Romeis et al., 1999) and a MAP kinase kinase (Xing et al., 2001) has been demonstrated in tobacco and tomato, respectively.

Although our experiments indicate that SA confers systemic induction of *SFR2* transcription, we cannot rule out the involvement of other pathways in the wound-response of the *SFR2* promoter. In particular, it should be noted that plants expressing *NahG* (in contrast to *sid2* mutants which have very low levels of SA due to a mutation in a plastidic isochorismate synthase involved in SA biosynthesis) exhibit not only reduced SA levels but are also affected in the accumulation of ethylene and jasmonic acid as well as in the synthesis of the phytoalexin camalexin (Heck et al., 2003). This may indicate that minimal levels of SA are required to potentiate other defence pathways. Alternatively, NahG activity may alter signalling molecules, as suggested by the similarity of gene expression patterns in *NahG* and *pad4* plants (Glazebrook et al, 2003). PAD4, a protein containing a lipase domain, has been assigned a pleiotropic regulatory function affecting camalexin biosynthesis and SA accumulation (Shah, 2003).

We considered the possibility that SA might mediate wound induction of *SFR2* indirectly, acting downstream of a more classical wound response signal, but this is unlikely because *SFR2* is induced very rapidly in response to wounding (transcripts start to accumulate 45 min after wounding; Pastuglia et al., 1997). It is possible, however, that SA is not directly involved in

induction but rather that basal levels of SA are required for the activation of *SFR2* transcription via a wound signalling pathway.

It will be interesting to further investigate the role of JA in the response of *SFR2* to wounding . *SFR2* transcripts do not accumulate following a local treatment with JA (Pastuglia et al., 1997) but it is not known if this molecule plays a role in systemic induction of *SFR2*. In general, the local wound response in *Arabidopsis* is thought to be mediated by oligogalacturonides whereas JA mediates the systemic response. However, JA-independent systemic wound response pathways have also been postulated (LeBrasseur et al., 2002). JA-insensitive mutants such as *coi1* could be used to further investigate the role of this molecule in *SFR2* wound induction.

To further analyse the mechanism of regulation of *SFR2* by SA, we searched the promoter region of this gene for elements that are required for the SA response. The promoter was found to have a complex structure. Deletion of the region between -504 bp and -447 bp consistently lead to a complete loss of SA inducibility, indicating that this region of the promoter is essential for the SA response. However, upstream of this major regulatory element, additional positive and negative regulatory elements were found in the regions -1295 bp to -740 bp and -740 bp to -612 bp, respectively.

Comparison of the *SFR2* promoter region with that of its *Arabidopsis* homologue, *ARK3*, indicated that these sequences are highly diverged. Only a short region of similarity was found and this region of shared similarity was located within the -504 bp to -447 bp region of the *SFR2* promoter required for induction by SA. The conservation of this region, which contains two putative W box elements, in the two species strongly indicates that it has a functional role. Further analysis of this region by creating specific deletions and by fusing multimers of the region to a minimal promoter indicated that it is essential for full induction of the *SFR2* promoter by SA.

However, this region alone is not sufficient to mediate induction and it appears to function essentially as an enhancer. Similar results have been reported by Du and Chen (2000), who observed that mutations in W boxes resulted in a drastic decrease in gene expression under uninduced conditions.

The fact that the factors which bound to the *SFR2* promoter element in the EMSA analysis were present in untreated as well as SA-treated plant extracts is also consistent with this element acting as an enhancer. These factors do not bind efficiently to the as-1 element indicating that they are more likely to be WRKY transcription factors than members of the bZIP family. The presence of DNA-binding activities in untreated nuclear extracts has previously been demonstrated in EMSA experiments using a probe with TTGACC repeats (Dong et al., 2003). However, in contrast to the factors identified by Dong et al., the abundance of the binding proteins detected with our probe does not increase following SA treatment. Again, this is consistent with the promoter element functioning as an enhancer. In summary, the region of the *SFR2* promoter containing the putative W boxes between –504 and –447bp plays an important role in the induction of this gene by SA but it probably does not function alone. Full induction is likely to require a combination of elements in the *SFR2* promoter, possibly including additional W boxes found downstream of position –447 bp. These W boxes may also be involved in the wound-response of the *SFR2* promoter since WRKY transcription factors have been shown to accumulate rapidly and systemically upon wounding (Hara et al., 2000).

In conclusion, the rapid, SA-dependant accumulation of *SFR2* mRNA to high levels in response to bacterial infection and wounding, indicates that this gene may be an important component of the plant's response to these stimuli. Moreover, the observation that, in *Arabidopsis*, transcripts for several PRKs accumulate following SA treatment (Ohtake et al., 2000) suggests that SFR2 may be only one of a battery of receptors that are induced in response to external

aggression and that the abundance of these receptors may be modified in a coordinated manner via changes in endogenous SA levels.

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Figure legends

Fig. 1. Wound induction of *SFR2* is systemic and requires an SA signal. β-glucuronidase activity was assayed in extracts of rosette leaves of *Arabidopsis* lines carrying either a chimeric gene construct consisting of 1516 bp of the *SFR2* promoter region fused to the *gusA* reporter gene (*SFR2p::gusA*) or the same chimeric gene plus the *NahG* transgene (*SFR2p::gusA* x *NahG*). To measure the local response, leaves were either harvested before wounding, as a control, or one day after wounding. The systemic response was measured by harvesting leaves adjacent to the wounded leaf either one or seven days after treatment. Average fold inductions are indicated by filled circles. Error bars represent standard deviations.

Fig. 2. Deletion analysis of the *SFR2* promoter. A, Fragments of the *SFR2* promoter representing a 5' deletion series, were fused to the *gusA* reporter gene. Lengths of deletion fragments are indicated in bp on the right. Numbering is relative to the first base of the translation start codon of

SFR2. The transcription start site is indicated by an angled arrow. Putative CAAT and TATA boxes, described by Pastuglia *et al.* (1997), and the ATG initiation codon are represented by vertical bars within the box. Horizontal arrows indicate the position of repeated sequences. B, Average values of β-glucuronidase activity for the indicated promoter constructs in primary transformants before (open bars) and after treatment *in vitro* with either 50 mM phosphate buffer pH 7 (hatched bars) or 5 mM SA in 50 mM phosphate buffer pH 7 (grey bars). C, Average values of β-glucuronidase activity for the indicated promoter constructs in T_2 plants after treatment *in vivo* with either 50 mM phosphate buffer pH 7 (hatched bars) or 1 mM SA in 50 mM phosphate buffer pH 7 (grey bars). For each transgenic line, fold induction was calculated as the ratio of the activity measured after treatment with SA in phosphate buffer and the activity obtained with phosphate buffer alone as a control. Average fold induction is the mean value of the fold inductions calculated for individual lines and is indicated by a filled circle for each construct. The number of transgenic lines analysed for each construct is given (n).

Fig. 3. Comparison of the *Brassica* SFR and the *Arabidopsis* ARK genes. A, Phylogenetic relationships and comparison of the expression patterns of the SFR and ARK genes. A rooted neighbour-joining tree was constructed from an alignment of the entire deduced polypeptides. The *S* locus receptor kinase (SRK_3; Delorme *et al*., 1995) was included as an outgroup. Bootstrap values were calculated from 1000 replicates. The expression pattern of each gene, based on data from Pastuglia *et al*. (1997), Tobias *et al*. (1992), Dwyer *et al*. (1994), Ohtake *et al*. (2000), Pastuglia *et al*. (2002) and this study, is summarised to the right. NA, data not available. B, Average β-glucuronidase activity in leaves of transgenic *Arabidopsis* plants carrying chimeric gene constructs consisting of either 1428 bp or 429 bp of the region upstream of the *ARK3*

coding sequence fused to the *gusA* reporter gene. C: control, untreated leaves; PB: control leaves sprayed with 1 mM phosphate buffer; 1 mM SA: leaves sprayed with 1 mM SA. Leaves were harvested 24 h after treatment. Average fold inductions compared with phosphate buffer treated controls are indicated by filled circles. Nine and 17 transgenic plants were analysed for 1428 bp and the 429 bp promoter construct, respectively. C, Alignment of the -520 to -449 region of the *SFR2* promoter with the -430 to -363 region of the *ARK3* promoter. The -520 to -449 region of the *SFR2* promoter is part of a long, imperfect repeat and the corresponding duplicated region (-414 to -343) is also shown. Identical bases are highlighted with black boxes. Two TGAC motifs in the *SFR2* promoter are underlined.

Fig. 4. Functional analysis of the conserved region of the *SFR2* promoter. A, Effect of deleting the conserved region on *SFR2* promoter activity in whole plants. β-glucuronidase activities in leaves of transgenic *Arabidopsis* plants carrying chimeric gene constructs consisting of either the 1516 bp *SFR2* promoter fused to *gusA* (1516) or the same promoter with either 57 (1516Δ57) or 12 bp (1516Δ12) of the conserved region deleted (see text for details). Three to 4 pools (25 plants/pool) of 8 lines were analysed for each construct. Open bars, no treatment; black bars, 24 h after removing the first leaf; hatched bars, 50 mM phosphate buffer pH 7; grey bars, 1 mM SA in 50 mM phosphate buffer pH 7. B, Effect of the conserved region on expression from a minimal promoter in detached leaves. Four direct repeats of a 29 bp region of the conserved region (4xWT-46), a mutant form of this region (4xMUT-46), the *as-1* element (4xAS1-46) or no element (-46) were placed upstream of the *35S* -46 minimal promoter and the *gusA* reporter gene. The repeated motif is shown in each case. Wild type or mutated TGAC motifs are underlined. T_1 plants of 30 to 40 lines were analysed for each construct. Open bars, no treatment; hatched bars,

50 mM phosphate buffer pH 7; grey bars, 1 mM SA in 50 mM phosphate buffer pH 7. Error bars represent standard deviations.

Fig. 5. Cellular factors bind specifically to the conserved region of the *SFR2* promoter. EMSA analysis was carried out using four direct repeats of the -487 to -469 region of the *SFR2* promoter (4xWT, see Figure 4B) as a probe and cellular extract from either control leaves (0 mM SA) or from leaves sprayed with 1 mM SA. Double stranded oligonucleotide competitors were added to the assays at the molar ratios indicated. The competitors were composed of four direct repeats of either the -487 to -469 region of the *SFR2* promoter (4xWT), a mutant form of this sequence in which the TGAC motifs had been modified (4xMUT) or the as-1 element of the 35S promoter. The sequences of the oligonucleotide competitors were identical to the sequences shown in Figure 4B. Complexes are indicated by horizontal bars.