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Rapid Induction by Wounding and Bacterial Infection of an *S* Gene Family Receptor-like Kinase Gene in *Brassica oleracea*

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A receptor-like kinase, SRK, has been implicated in the autoincompatible response that leads to the rejection of self-pollen in Brassica plants. SRK is encoded by one member of a multigene family, the *S* gene family, which includes several receptor-like kinase genes with patterns of expression very different from that of *SRK* but of unknown function. Here, we report the characterization of a novel member of the Brassica *S* gene family, *SFR2*. RNA gel blot analysis demonstrated that *SFR2* mRNA accumulated rapidly in response both to wounding and to infiltration with either of two bacteria: *Xanthomonas campestris*, a pathogen, and *Escherichia coli*, a saprophyte. *SFR2* mRNA also accumulated rapidly after treatment with salicylic acid, a molecule that has been implicated in plant defense response signaling pathways. A *SFR2* promoter and reporter gene fusion was introduced into tobacco and was shown to be induced by bacteria of another genus, *Ralstonia (Pseudomonas) solanacearum*. The accumulation of *SFR2* mRNA in response to wounding and pathogen invasion is typical of a gene involved in the defense responses of the plant. The rapidity of *SFR2* mRNA accumulation is consistent with SFR2 playing a role in the signal transduction pathway that leads to induction of plant defense proteins, such as pathogenesis-related proteins or enzymes of phenylpropanoid metabolism.

INTRODUCTION

Cell surface receptors with an intrinsic protein kinase activity have been shown to play important roles in signal perception and transduction in animals. A number of similar genes predicted to encode receptor kinases have been identified in plants. In a few cases, protein kinase activity has been demonstrated, and the protein product has been shown to be anchored in the membrane (Chang et al., 1992; Goring and Rothstein, 1992; Mu et al., 1994; Delorme et al., 1995; Stein et al., 1996). Five different classes of plant receptor-like kinases have been identified, as defined by the predicted amino acid sequences of their extracellular domains. The first class consists of receptor-like kinases of the *S* gene family and is so designated because the members of this family share homology with the *S* locus glycoprotein (SLG) of Brassica (Nasrallah et al., 1994b). This class includes the *S* locus receptor kinase (SRK) of Brassica (Stein et al., 1991), ZmPK1 of maize (for *Zea mays* protein kinase 1; Walker and Zhang, 1990), ARK1, ARK2, and ARK3 of Arabidopsis (ARK for Arabidopsis receptor kinase; Tobias et al., 1992; Dwyer et al., 1994), and OsPK10 of rice (for *Oryza sativa* protein kinase 10; Zhao et al., 1994). The four other classes include receptor-like kinases predicted to possess extracellular domains containing leucine-rich repeats (e.g, TMK1 for transmembrane kinase 1, PRK1 for pollen receptor kinase 1, and Xa21; Chang et al., 1992; Mu et al., 1994; Song et al., 1995), epidermal growth factor repeats (WAK1 for wall-associated kinase 1; Kohorn et al., 1992), a thaumatin-like domain (PR5K for pathogenesis-related 5 kinase; Wang et al., 1996), or a lectin-like domain (Ath.lecRK1 and LRK1 for *Arabidopsis thaliana* lectin receptor kinase 1 and lectin receptor kinase 1, respectively; Hervé et al., 1996; Swarup et al., 1996).

Plant receptor-like kinase genes have been isolated from a wide range of both dicotyledonous and monocotyledonous plant species. They show markedly different patterns of expression, with some genes being expressed in specific reproductive tissues (*PRK1* and *SRK*) and others predominantly in vegetative tissues (*ARK* genes), whereas others show developmentally regulated expression in both floral and vegetative tissues (*ZmPK1* and *TMK1*).

Xa21 and *PRK1* are the only members of the plant

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receptor-like kinase family to have been unequivocally assigned a role. Rice transformation experiments have shown that *Xa21* confers resistance to *Xanthomonas oryzae* pv *oryzae* race 6. Antisense inhibition of *PRK1* expression in transgenic *Petunia inflata* has demonstrated that this gene is essential for postmeiotic development of pollen (Lee et al., 1996).

Within the *S* gene subfamily of receptor-like kinases, *SRK* is thought to be involved in the pollen–pistil recognition step of the self-incompatibility response in Brassica (McCubbin and Kao, 1996). *SRK*'s proposed role in self-incompatibility is based on its stigma-specific pattern of expression, its location at the *S* locus (which controls self-incompatibility), and its polymorphic nature as well as on the existence of self-compatible Brassica plants that also possess mutated *SRK* genes (Goring et al., 1993; Nasrallah et al., 1994a). Apart from XA21, PRK1, and SRK, very little information is available concerning the roles of receptor-like kinases in plants; in particular, the functions of other receptor-like kinases of the *S* gene family are not known.

It is likely that *S* gene family receptor-like kinases expressed in vegetative tissues are involved in cell–cell communication systems analogous to the self-incompatible pollen–pistil interaction, except that they probably regulate very different developmental or metabolic pathways. A detailed study using the β-glucuronidase (*GUS*) reporter gene fused to the promoters of *ARK2* and *ARK3* has shown that these receptor-like kinase genes exhibit highly specific patterns of temporal and tissue-specific regulation (Dwyer et al., 1994). Based on these observations, these authors suggest that *ARK2* and *ARK3* may function in specific aspects of plant growth or development but that their specific role in the plant is not yet known. Another possible function of *S* gene family members in vegetative tissues is suggested by the numerous similarities between self-incompatibility and the plant's response to attack by a pathogen (Hodgkin et al., 1988). In both phenomena, genetically controlled recognition systems exist that allow one cell to recognize another, leading to the induction of the response: self-incompatibility occurs when the pollen and the papilla cell express the same *S* locus haplotype (Nasrallah et al., 1994b), whereas disease resistance in many cases depends on the pathogen expressing an avirulence (*avr*) gene that corresponds to a resistance gene present in the plant host (Flor, 1971). From a morphological point of view, the comparison with self-incompatibility is particularly vivid for a fungal pathogen; in both cases, an elongated cell (a pollen tube or a germ tube) emerges from a sporelike structure (a pollen grain or a fungal spore) on the surface of the host plant and penetrates by growing within or between cell walls (Dickinson, 1994).

In this study, we show that a novel member of the *S* gene family encoding a receptor-like kinase is induced by a range of stimuli that induce plant defense genes. These stimuli include both wounding and bacterial attack. These results indicate that this gene may play a role in the response of the plant to mechanical and biological attack.

RESULTS

Cloning and Sequence Analysis of the *SFR2* Receptor-like Kinase Gene

A polymerase chain reaction probe, generated by amplification of genomic DNA sequences with oligonucleotides corresponding to conserved regions of the SLG_{29} gene (Trick and Flavell, 1989), was used to screen a leaf cDNA library. Five cDNAs hybridized with the probe, and these were shown to represent three different genes, by use of restriction mapping and DNA sequencing. The complete cDNA sequence corresponding to one of these genes, designated *SFR2* (for *S* gene family receptor 2), was reconstructed from two clones. One of the clones was not full length but included the poly(A) tail, and the second included the 5′ untranslated region but lacked part of the 3′ untranslated region due to a recombination event, which apparently had occurred during or after the construction of the library. Based on this sequence, *SFR2* is predicted to encode a membrane-spanning, receptor-like kinase with a structure very similar to that of SRK, possessing an extracellular *S* domain, a single membrane-spanning domain, and an intracellular kinase domain.

The deduced amino acid sequences of both the *S* domain and the kinase domain of SFR2 (EMBL accession number X98520) were compared with those of several other members of the *S* gene family from both Brassica and Arabidopsis (Table 1). In all of the pairwise comparisons, the kinase domains were more similar than were the *S* domains; however, in general, when the kinase domains were more closely related, the *S* domains were as well, and vice versa. Interestingly, SFR2 was more similar to the ARK receptor-like kinases of Arabidopsis than to the Brassica receptor-like kinase SRK. It is possible that *SFR2* is the Brassica equivalent of one of the *ARK* genes. Two other receptor-like kinases from Arabidopsis, RLK1 and RLK4, are more distantly related to SFR2 (Table 1), as are four putative Brassica receptor-like kinases identified by Kumar and Trick (1993; data not shown). In addition, an *SFR2 S* domain probe hybridized most strongly with genomic DNA fragments corresponding to the *ARK* genes in a genomic gel blot of Arabidopsis DNA (R. Swarup and J.M. Cock, unpublished results).

Tissue-Specific Expression of the *SFR2* Gene

RNA gel blot analysis was used to determine the abundance of *SFR2* transcripts in a number of floral and vegetative tissues (Figure 1). A 320-bp, 3′ untranslated region probe was used for these experiments. This probe was shown to be gene specific by hybridization to a gel blot of *Brassica oleracea* genomic DNA digested with several restriction enzymes (data not shown). The *SFR2* probe detected a major tran-

Table 1. Comparison of the Percentage of Amino Acid Similarity[a] between the Sequence of SFR2 and Those of Selected Receptor-like Kinases of the S Gene Family[b]

	SFR2	SRK_6	ARK1	ARK2	ARK3	RLK1	RLK4
SFR2		57.7	67.4	67.3	79.6	18.3	25.9
SRK_6	67.8		56.1	52.9	59.3	16.5	21.4
ARK1	78.2	68.6		77.2	69.7	15.9	22.6
ARK2	77.8	67.3	92.9		68.7	17.4	20.3
ARK3	83.4	68.8	79.4	80.9		17.7	23.8
RLK1	25.4	25.1	25.7	25.7	28.6		15.4
RLK4	26.4	26.4	27.5	28.5	27.0	35.3	

[a] The results of the comparisons are expressed as percentage similarity that is calculated as follows: 100 times the number of matched amino acids divided by the sum of the length in amino acids of the aligned region, plus the number of gaps introduced to optimize the alignment.
[b] Above the diagonal: similarity between pairs of S domains; below the diagonal: similarity between pairs of kinase domains. SRK_6 is from *B. oleracea* (S_6 haplotype; Stein et al., 1991). ARK1, ARK2, and ARK3 (Tobias et al., 1992; Dwyer et al., 1994) and RLK1 and RLK4 (Walker, 1993) are from Arabidopsis.

script of 2.8 kb and two less abundant transcripts of 1.3 and 1.6 kb. The size of the 2.8-kb transcript is consistent with it being the full-length, fully spliced transcript corresponding to the *SFR2* cDNA. The 1.3- and 1.6-kb bands may represent alternative transcripts of the *SFR2* gene generated by alternative splicing. Multiple transcripts have been reported for the closely related *SRK* gene in *B. oleracea* (Stein et al., 1991; Delorme et al., 1995).

The 2.8-kb transcript accumulated in a developmentally regulated, tissue-specific manner and was most abundant in flower buds. mRNA levels were at their highest when the buds were 8 mm in length (Figure 1A). When the individual organs of the flower were analyzed, *SFR2* mRNA was most abundant in anthers from 5- to 7-mm-long flower buds. There was also an accumulation of *SFR2* mRNA in stigmas but at a later stage, with the highest levels being detected in stigmas from open flowers (Figure 1B). No *SFR2* mRNA was detected in petals, sepals, or ovary tissue at the open flower stage, but a very low abundance of *SFR2* mRNA was detected in the seeds throughout the development of the silique. In vegetative tissues, *SFR2* mRNA was not detected in 3-day-old seedlings but was present both in leaves of field-cultivated plants and in roots of 3-week-old plants, with the highest level being in the leaves (Figure 1C).

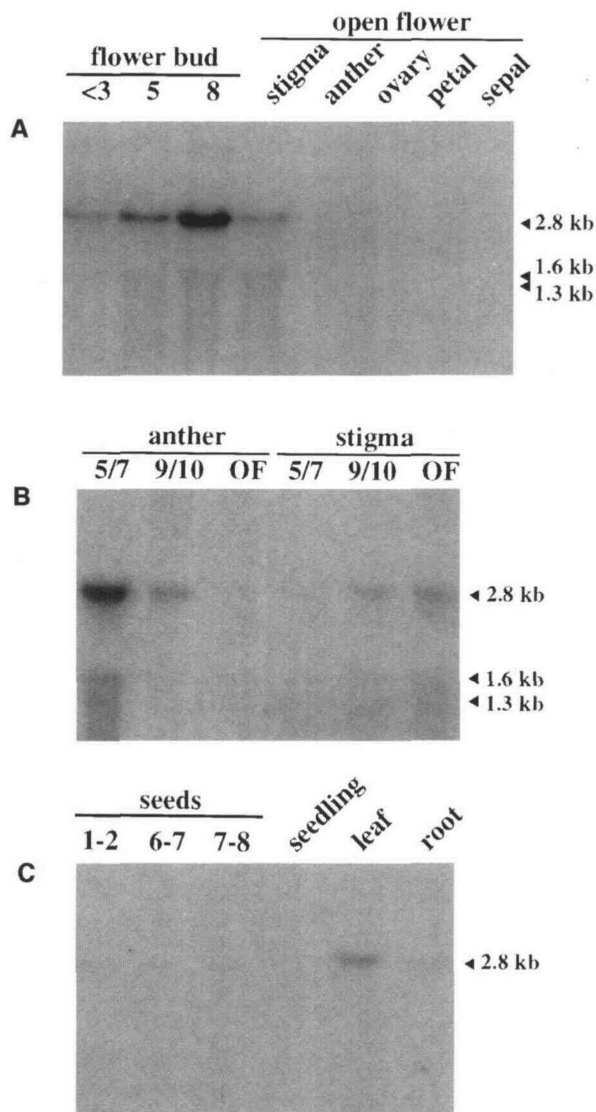

Figure 1. Tissue-Specific and Developmental Regulation of *SFR2* mRNA Accumulation in *B. oleracea*.

RNA gel blot analysis of *SFR2* mRNA in a range of tissues at different stages of development was conducted.
(A) Flower buds of different lengths (given in millimeters) and stigma, anther, ovary, petal, and sepal tissue from open flowers.
(B) Anthers and stigmas harvested from flower buds of different lengths (5 to 7 and 9 to 10 mm) or from open flowers (OF).
(C) Seeds from siliques that were 1 to 2, 6 to 7, or 7 to 8 cm long, 3-day-old seedlings, leaves from field-cultivated plants, and root tissue from 3-week-old plants.
The lengths of the *SFR2* transcripts are indicated at right in kilobases (kb).

Accumulation of *SFR2* mRNA in Response to Wounding and Bacterial Infection

To determine whether the expression of *SFR2* could be influenced by an external stimulus, leaves of *B. oleracea*

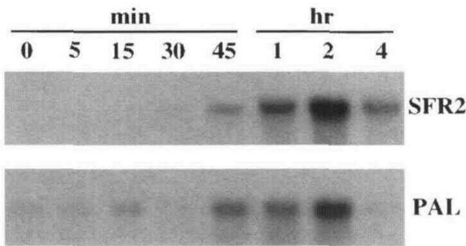

Figure 2. Wound-Inducible Accumulation of *SFR2* and *PAL* mRNA in *B. oleracea* Leaves.

Fully expanded leaves were wounded by rubbing the undersides with abrasive paper. RNA gel was prepared at different times after treatment (0 to 4 hr), and an RNA gel blot was probed with either the *SFR2* probe (SFR2) or a *PAL10* probe (PAL; Dong et al., 1991).

plants were wounded, and the abundance of *SFR2* mRNA was assayed by using RNA gel blots. Figure 2 shows that *SFR2* transcripts began to accumulate between 15 and 30 min after wounding, reaching a maximum within 2 hr. *SFR2* mRNA therefore accumulated rapidly and transiently in wounded leaves.

The kinetics of accumulation of *SFR2* mRNA after mechanical wounding was compared with that of phenylalanine ammonia-lyase (*PAL*) mRNA, which is known to accumulate early during the defense response. *PAL* is a well-characterized defense gene that encodes the first enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase. *SFR2* and *PAL* mRNAs were shown to start accumulating at approximately the same time after the wound stimulus (Figure 2).

An experiment was then performed to determine whether *SFR2* mRNA also accumulated in response to invasion of the plant by a bacterial pathogen. *B. oleracea* leaves were infiltrated with two strains of *X. campestris* pv *campestris*, one wild type (pathogenic) and the second mutated in the *hrp* gene cluster, which is required both for pathogenicity in the plant host and for induction of the hypersensitive response (HR) in non-host plants (Arlat et al., 1991). The *hrp* mutant strain elicited no visible symptoms. Water was also infiltrated as a control. Figures 3A and 3B show that *SFR2* mRNA accumulated in response to both bacterial strains. At

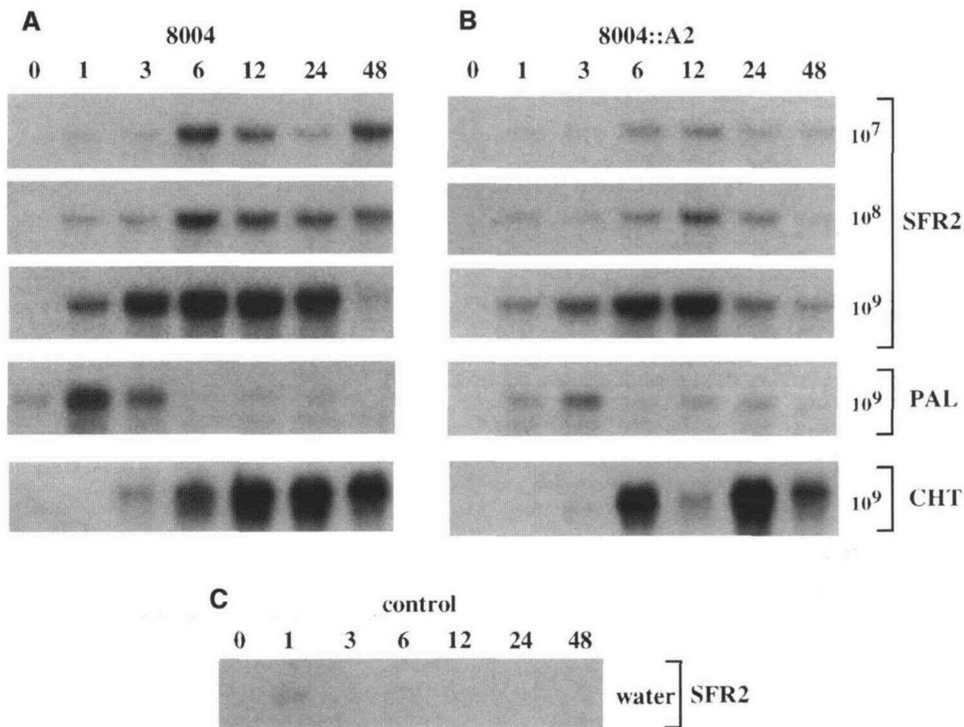

Figure 3. Induction of *SFR2*, *PAL*, and Chitinase mRNA Accumulation after Infection of *B. oleracea* Leaves with the Bacterial Pathogen *X. c. campestris.*

(A) and **(B)** Fully expanded leaves infiltrated with either wild-type (8004 strain) or *hrp* mutant (8004::A2 strain) bacteria suspended at a concentration of 10^7, 10^8, and 10^9 cells mL^{-1}. RNA was prepared from the infiltrated tissue at different times after infection (0 to 48 hr), and an RNA gel blot was probed with the *SFR2* probe, a *PAL10* probe (PAL), and a basic chitinase probe (CHT; Rasmussen et al., 1992).

(C) *Fully expanded leaves infiltrated with water alone.*

Time in hours is indicated above the gels.

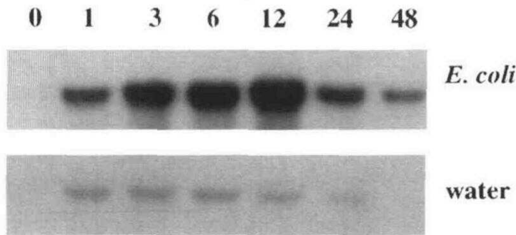

Figure 4. *SFR2* mRNA Accumulation in *B. oleracea* Leaves Infiltrated with the Saprophytic Bacterium *E. coli.*

Fully expanded leaves were infiltrated with *E. coli* DH5α at a concentration of 5 × 10^8 cells mL^{-1} or with water alone. Time in hours is indicated above the gels.

the highest concentration of wild-type bacteria, *SFR2* mRNA had already started to accumulate 1 hr after infiltration and by 6 hr was 66-fold more abundant than in the control. Again, the accumulation of *SFR2* mRNA was transient, and at the highest concentration of bacteria, mRNA abundance had returned to the same level as in the control after 48 hr. *SFR2* mRNA accumulated later when a lower concentration of bacteria was infiltrated into the leaves. No significant accumulation of *SFR2* mRNA was seen when the leaves were infiltrated with water alone (Figure 3C), indicating that the mRNA accumulation induced by the bacteria was not due to wounding of the leaf during infiltration.

Hybridizations with *PAL* and chitinase probes demonstrated that, like the *SFR2* gene, these two defense-related genes were induced in leaves infiltrated with either the pathogenic (8004) or the nonpathogenic (8004::A2) strain. Similar results have been reported for these two genes in bean after infiltration with a *Pseudomonas syringae* pv *tabaci hrp* mutant (Jakobek and Lindgren, 1993). In their study, Jakobek and Lindgren (1993) also reported that a saprophytic bacterium, such as *Escherichia coli*, was capable of inducing an accumulation of *PAL* and chitinase mRNAs. Therefore, we tested whether *E. coli* was able to induce accumulation of *SFR2* mRNA. Figure 4 shows that *SFR2* mRNA accumulated in *B. oleracea* leaves infiltrated with *E. coli*.

Several plant-derived chemicals have been shown to induce some of the responses that follow wounding and/or invasion by a pathogen. Three of these molecules, salicylic acid (SA), abscisic acid (ABA), and methyl jasmonate (MeJA), were infiltrated into *B. oleracea* leaves, and changes in *SFR2* mRNA abundance were analyzed (Figure 5). A rapid accumulation of *SFR2* mRNA, peaking between 3 and 6 hr after treatment, was observed in response to 1 mM SA. Recent studies have suggested that SA intervenes in the plant's defense response by inhibiting a catalase (the SA binding protein), leading to increased H_2O_2 levels and to the activation of defense-related genes (Chen et al., 1993). Using a range of SA analogs, Chen et al. (1993) demonstrated a correlation between the ability to bind the SA binding protein and inhibit its catalase activity and the effect on pathogenesis-related protein induction. We tested the activity of two analogs, 4-hydroxybenzoic acid and 4-aminosalicylic acid, that are unable to bind the catalase (Chen et al., 1993; Sanchez-Casas and Klessig, 1994). Neither analog induced a significant accumulation of *SFR2* mRNA or chitinase mRNA in *B. oleracea* leaves compared with the control treatments (data not shown).

In contrast to the response to SA, no significant accumulation of *SFR2* mRNA was seen after infiltration of ABA or MeJA. To confirm that the infiltrated tissue was correctly treated with these chemicals, we hybridized radiolabeled probes corresponding to genes known to be induced in the presence of MeJA (*Atvsp* for *A. thaliana* vegetative storage protein; Berger et al., 1995) or ABA (*AtDi21* for *A. thaliana* drought-induced 21; Gosti et al, 1995) with RNA gel blots corresponding to the middle and bottom gels, respectively, shown in Figure 5. In both cases, mRNA accumulated after treatment (data not shown).

Analysis of the *SFR2* Promoter Region and the Expression of an *SFR2::uidA* Reporter Gene Fusion in Transgenic Tobacco

A 320-bp fragment corresponding to the 3′ untranslated region of the *SFR2* cDNA was used as a probe to isolate clones carrying the SFR2 gene from a genomic library. The DNA sequence was determined for a region of 1480 bp upstream of the ATG initiation codon (Figure 6). There was no obvious similarity between the *SFR2* promoter and those of other members of the *S* gene family; in particular, no homology was detected with the five conserved "boxes" present

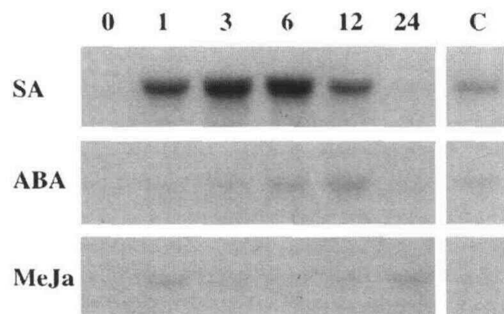

Figure 5. Effects of Chemical Inducers on *SFR2* mRNA Abundance.

Fully expanded leaves were infiltrated with 1 mM SA, 100 μM ABA, or 50 μM MeJA. RNA was prepared at different times after treatment (0 to 24 hr), and RNA blots were probed with the *SFR2* probe. Control plants (C) were treated with the carrying solution minus the inducer (5 mM phosphate buffer, pH 7, for SA; 5 mM phosphate buffer, pH 7, and 0.053% ethanol for ABA; and water for MeJA), and the tissue was harvested after 6 hr. Time in hours is indicated above the gels.

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TCTTTTGATTAGATGATTTTATAAATTAGAATAAACACAGTCGTGGGGTTGGAGCAATTGTTAAATTAGAATATAATTTT -1401
TTTTTTTTAAATTAATTCGAAATTTGGATTAGTCAAAAAAAAAATTCGAAATTTGGAAGGAACAAACTGGAGGCTGGTTG -1321
                                                                      Box I
  TCA-1
GAGAGAAGAATAGTTAAGGTAAACGTGGAACGACACCCTTCTATTTTGTTTTCGGGGAATGCGGAATTAAATTAAAGTTG -1241
                   G box   TGA-1
GTTCTGTTGGTGAACTGGAGTTATTCATTGGGAAGGAAACAAAAATCTCGAAAAAGAGCTCTCTTCAGAGCATATTCAAT -1161
  GARE
GAAATTTCTAATAGATTCTTTTTTCTTGAACCACCTAATAGATTCTTCAAACTTTACAAAATGAAAAAAAAAAAGAATTA -1081
GAACATCACTATGTCCAGAATGAAGAAAATGTTTTAAAAAACTATTGAAATATAAAAACCAATTATATATTAAACGAGTA -1001
                          Hsp27                      ELI box 3
                                                                 WUN
TTTCATGTTTTCGGGACTTTTTTAAAGATTCAATTATTTTTAATGTTTAATATTTTTTTAAAAAATTTCCTATAAAACGA  -921
                                                               HSE
GATTCCTTTCTGAAACGTTCAAACTAAAGAAAATTCGCTTAAAAACTTTAGAAACTCTTTCGTTACATGTCTTCTTTTTA  -841
   HSE     HSE              HSE           HSE      HSE
GTGGTCTTCGCTTCTTAATTAGCATATAAATATTTTAACTTTTAGTCCCTGGCCATCGAGGTTGAACTTATCATAAACAT  -761
TCCCTTTTACTCTCTGTCTTTAGATATGTAAGTAGTTTAAGAAAAAAAATGTTTATTTCACAGATGTTTACATAATCTAA  -681
AACATTTTTTATATTTATTACATGTTGTATGATCAATTAAATAATTCATATTTTTTTTTATTATTGGTTGAATTTATGTA  -601
                                                             ELI box 3
TTAAATGCTATTTTTTAAAAAGTAAATTTTTTTTTAATATTCACAATTTTAACTAAAACTACTTACGAATAAAAACAAAA  -521
                                                             PI WUN
GATAATCACTCCCATACATACAAACCGGTTGACTGACTATTGGCTGGAAAAAAAATCAGAAGAGGAAGTAAAGTTTAACT  -441
                                                         TCA-1
AAAACTACTTGCAGATAAAAACAGAGGAGTATAACTCTCATACATACAAACCGGTTGACTGACTATTGGCTGGAAAAAAA  -361
                    GARE
ATCAGAAAAGGAAGTAAAGTCATTTATGTTAACTGAATAACTGTGATATTTAATTTATTAACAAAGATAACGCGTAGAAA  -281
    TCA-1
CGAAAGGAAGCCTGACAAAATAAATAAAAACAACTTTCTTAACACGTAGGTCAACTCTCAATCCGACTGTACATAACATA  -201
                                         G box
ACTACATATAAGTATGTTCAAACCTGGAAGACCAAGAAAATTAAAAAAGCATAGAAAGGAATTTATGTTGATAATCCAAG  -121
TAAATAAAAGTTCATGCATTTAACACAAAACATGTCCGATATCTCCAATGAAAAATCAAATCCTCAAATTTACATATAAT   -41
                                             CAAT                         TATA
TTTCTCAACAAAGAGAGAGAGAAAAAACAAGAACACAAGAATG                                          +2
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Figure 6. Nucleotide Sequence of the Promoter Region of *SFR2*.

Numbering is relative to the first base of the ATG codon, which is shown in italics. The transcription start site, as determined by ribonuclease protection analysis, is indicated with a bent arrow. Motifs with significant similarity to previously identified *cis*-acting elements are underlined or overlined. These include CAAT and TATA boxes, an SA response element (TCA-1; Goldsbrough et al., 1993), an elicitor-responsive element (ELI box 3; Ohl et al., 1990), wound-responsive elements (WUN; Matton et al., 1993; and PI WUN; Palm et al., 1990), a MeJA-responsive element (box 1; Mason et al., 1993), the heat shock element (HSE; Gurley and Key, 1991), a G-box element (G box; Nagao et al., 1993), an auxin-responsive element (TGA-1; Nagao et al., 1993), and a gibberellin-responsive element (GARE; Sutliff et al., 1993). A short region of near identity to part of the heat shock protein 27 promoter (*Hsp27*) is also underlined. Also shown is a region of the promoter where four sequences (indicated by arrows) are repeated directly downstream. The EMBL accession number for the *SFR2* promoter region is X98521.

in the promoter regions of *SRK*, *SLG*, and *SLR1* (for S locus-related 1; Dzelzkalns et al., 1993; Delorme et al., 1995). However, a number of sequences with significant similarity to previously characterized elements were identified, including elements identified in many inducible genes, such as the SA-responsive element TCA-1, the elicitor-responsive element ELI box 1, and the wound-responsive elements WUN and PI WUN (Figure 6).

To determine whether the *SFR2* promoter is able to direct inducible gene expression, we introduced a transcriptional fusion between the *SFR2* promoter and the *uidA* bacterial reporter gene (which encodes GUS) into tobacco by transformation, and the leaves of transgenic plants were subjected to a range of stimuli. Three different strains of *Ralstonia* (*Pseudomonas*) *solanacearum* were locally infiltrated into undetached leaves. Strain K60 caused the typical lethal wilting disease. Strain GMI1000 induced development of an HR on tobacco leaves within 18 to 24 hr after infiltration. No symptoms were observed with strain Δhrp, which carries a deletion in the *hrp* gene cluster required for elicitation of the HR response in tobacco (Boucher et al., 1985).

GUS activity was measured at different times after infiltration with the bacteria. Figure 7 shows that there was a low level of induction of expression of the gene fusion when the leaves were infiltrated with water but that gene expression significantly increased in the presence of bacteria. Strains Δhrp, K60, and GMI1000 all consistently induced expression of the gene fusion. The *SFR2* gene was therefore shown to respond to bacteria from two different genera, *Xanthomonas* and *Ralstonia*.

Histochemical detection of GUS was performed with leaves of transgenic plants locally infiltrated with the three *R. solanacearum* strains (Figures 8B to 8D). GUS activity was first detected in the infiltrated area 20 hr after infiltration with either the K60 or the Δhrp isolate. In the case of the GMI1000 isolate, GUS activity was first detected 12 hr after inoculation. After 18 hr, an HR developed, and GUS activity was restricted to cells surrounding the necrotic area.

The *SFR2*::*uidA* gene fusion was induced by infiltration with SA but not with ABA or with MeJA (data not shown). This result indicates that the *SFR2*::*uidA* gene fusion was regulated in tobacco in a manner similar to the endogenous *SFR2* gene in *B. oleracea*.

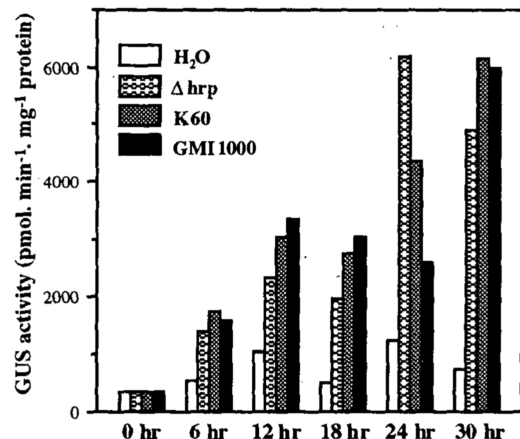

Figure 7. Time Course of *SFR2* Promoter Activation after Bacterial Infection in Transgenic Tobacco Plants.

GUS activity was analyzed in leaves locally infiltrated with water or with three different isolates of *R. solanacearum*: Δhrp (no visible symptoms), K60 (disease symptoms), or GMI1000 (HR).

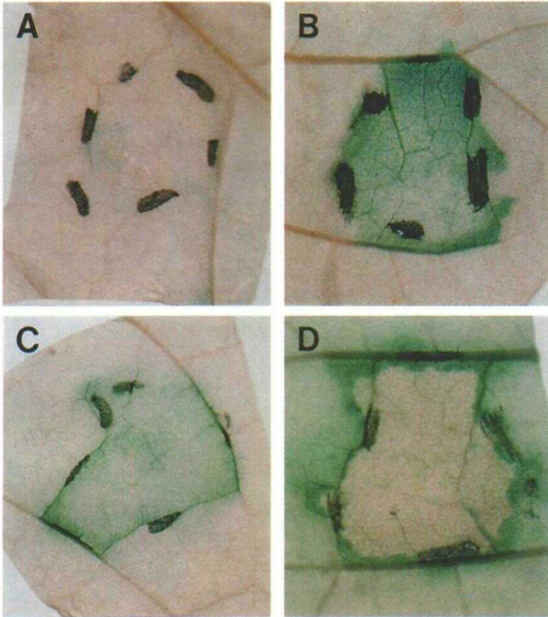

Figure 8. Histochemical Localization of *GUS* Gene Expression in Leaves after Bacterial Infiltration of Transgenic Tobacco Plants.

Tobacco leaves were locally infiltrated with water or with one of three *R. solanacearum* strains. The ringed areas indicate the borders of the infiltrated region.
(A) Leaf infiltrated with water.
(B) to **(D)** Leaves infiltrated with Δhrp (no visible symptoms), K60 (disease symptoms), and GMI1000 (HR), respectively.

DISCUSSION

In this study, we characterize the *SFR2* gene that encodes a putative receptor kinase of the *S* gene family in *B. oleracea*. The structure of the predicted protein is similar to that of other previously described members of the family in that it consists of an *S* domain, similar to that of SLG, and a kinase domain separated by a hydrophobic domain that is predicted to span the membrane. Sequence comparison (Table 1) indicates that *SFR2* is more closely related to the ARK family of receptor-like kinases in Arabidopsis than to other known receptor-like kinases from Brassica. The highest amino acid similarity was to ARK3, although the expression pattern of *SFR2* more closely resembles those of *ARK1* and *ARK2*, which are both expressed most strongly in the leaves.

Several treatments, including wounding, bacterial infection, and SA application, were shown to induce an accumulation of *SFR2* mRNA (Figures 2 to 5, 7, and 8). The response to SA was highly specific; *SFR2* mRNA did not accumulate in response to inactive analogs. There was no change in *SFR2* mRNA abundance in response to infiltration with MeJA, a substance that has been implicated in the signal transduction pathway leading to the induction of wound-induced genes, such as those encoding proteinase inhibitors (Farmer and Ryan, 1992). Although the expression of many wound-induced genes is stimulated by MeJA, there are exceptions (Farmer, 1994), indicating that wounding induces gene expression by at least two different signal transduction pathways.

The accumulation of *SFR2* mRNA in response to a stimulus was always transient, and mRNA abundance had returned to a basal level 6 to 24 hr after treatment, depending on the stimulus. The inducibility of *SFR2* in response to the stimuli tested is typical of a gene involved in plant defense and is consistent with the presence of sequences in the *SFR2* promoter region that are similar to regulatory elements implicated in the response to SA, elicitors, and wounding (Figure 6). Broad-response defense genes, such as those encoding PAL and chitinase, showed patterns of expression similar to *SFR2*. Transcripts corresponding to all three of these genes accumulated in response to wounding and to infiltration with *Xanthomonas* strains that either caused disease or elicited no visible symptoms (Figures 2 and 3).

Analysis of the spatial pattern of expression by using an *SFR2::uidA* reporter gene fusion in transgenic tobacco infiltrated with different strains of *R. solanacearum* showed that gene induction was initially restricted to the infiltrated region of the leaf (Figure 8). Later, in response to strain GMI1000, which caused an HR, expression extended slightly beyond the infiltrated area and corresponded to a region immediately surrounding the dying cells. In the case of the compatible interaction, it is likely that distal cells respond to the pathogen as the disease progresses, although this was not observed in the experiment performed here, which focused on the early stages of the infection process. Again, the pattern of expression observed with the gene fusion is typical of defense genes (Graham and Graham, 1991). The *SFR2::uidA* reporter gene fusion responded to three different strains of *R. solanacearum,* causing an HR response, disease, or no visible symptoms (Figures 7 and 8). Similarily, in *B. oleracea*, *SFR2* mRNA accumulated in the presence of both pathogenic and nonpathogenic strains of *X. c. campestris* (Figure 3) and in the presence of a saprophytic bacterium, *E. coli* (Figure 4); no response was observed when leaves were infiltrated with water. These data suggest that *SFR2* is induced by a wide range of bacteria and that it does not respond uniquely to pathogenic bacteria. Similar results have been reported for genes encoding PAL and chitinase in bean (Jakobek and Lindgren, 1993).

There is increasing evidence that protein kinases play a role in the plant's response both to wounding and to attack by pathogens. For example, there is evidence that a serine/threonine protein phosphorylation cascade constitutes part of the signal transduction pathway leading to resistance of tomato plants to *P. s. tabaci* strains carrying the *avrPto* avirulence gene (Martin et al., 1993; Zhou et al., 1995). Moreover, protein kinases resembling mammalian mitogen-activated protein (MAP) kinases have been shown to be activated

rapidly (in <5 min) after wounding (Usami et al., 1995) or elicitor treatment (Suzuki and Shinshi, 1995). mRNA corresponding to a MAP kinase homolog from tobacco has been shown to accumulate within 1 min of wounding (Seo et al., 1995). In mammals, recent evidence indicates that the MAP kinase pathway can mediate signal transduction from serine/threonine kinase receptors (Yamaguchi et al., 1995), although this connection has yet to be demonstrated in plants. Two other members of the receptor-like kinase supergene family have also been implicated in the defense response in plants: *Xa21*, which was identified as a resistance gene (Song et al., 1995), and *PR5K*, which is predicted to encode a receptor-like protein kinase with an extracellular domain related to a family of plant defense proteins (Wang et al., 1996).

The data presented here conclusively demonstrate that *SFR2* is inducible. It has recently been reported (A. Sasseen, P. Coello, and J.C. Walker, unpublished data) that *RLK4*, an *S* gene family receptor-like kinase gene from Arabidopsis, is increased in the presence of auxin, but to our knowledge there is no evidence that any of the other members of the receptor-like kinase superfamily, including *PR5K* and *Xa21* (or any of the more distantly related resistance genes; Staskawicz et al., 1995), are inducible. However, inducible gene expression has been observed for receptor kinases in animals. For example, both the epidermal growth factor receptor and the platelet-derived growth factor receptors are upregulated by their ligands (Clark et al., 1985; Eriksson et al., 1991). In both cases, however, mRNA accumulation seems primarily to be the result of post-transcriptional regulation, whereas in the case of *SFR2*, the inducibility of a *GUS* reporter gene fusion indicates transcriptional control. Transcriptional activation has been reported for the interleukin 2 receptor (Depper et al., 1985). Mammalian receptor kinases have been shown to be upregulated transiently after wounding (Antoniades et al., 1991; Wenczak et al., 1992). These receptors are thought to contribute in a number of ways to wound healing, for example, by allowing normally quiescent cells to respond to growth factors by proliferating to repair the wound.

SFR2 mRNA accumulated rapidly in response to the various treatments tested; for example, mRNA was already detected 15 to 30 min after wounding. The rapidity of *SFR2* induction was comparable to that of the early defense gene *PAL* (Figures 2 and 3). Nonetheless, assuming that SFR2 functions as a receptor, there is likely to be a significant lag between the initiation of *SFR2* induction and any response mediated by signal transduction via de novo synthesized SFR2. Hence, *SFR2* may not be involved in the initial steps of the defense response but rather may be implicated at a slightly later stage. Alternatively, it is possible that *SFR2* may function in the initial stages of the defense response if low levels of SFR2 protein are present in the tissue before it is challenged. In such tissues, accumulation of *SFR2* mRNA after wounding could then serve to amplify signal transduction via SFR2. It is interesting that a low level of *SFR2* mRNA was detected in leaves of field-grown plants (Figure 1).

SFR2 mRNA also accumulated in anthers and stigmas at specific developmental stages (Figure 1). Developmentally regulated expression has been reported for many defense genes, including pathogenesis-related genes (Castresana et al., 1990; Vogeli-Lange et al., 1994), genes involved in phenylpropanoid metabolism (Liang et al., 1989; Ohl et al., 1990), and a member of the *msr* (multiple stimulus response) gene family (Gough et al., 1995). All of these genes are strongly expressed during floral development. As has been suggested for these other defense genes, it is possible that SFR2 has a defense function at these specific development stages. Alternatively, SFR2 may control the expression of genes that are required both for defense and during development.

Evidence of a role for members of the *S* gene family in a wide range of processes within the plant, including pollen–pistil interactions (Stein et al., 1991; Nasrallah et al., 1994b), plant defense (this work), and development (Dwyer et al., 1994), is accumulating. These data suggest a model in which an ancestral recognition system has been adapted and diversified to undertake multiple roles in the plant. Preliminary investigations indicate that the *S* gene family includes a large number of receptor-like kinase genes, at least in Brassica (Kumar and Trick, 1993). Further characterization and functional analysis of the different members of this family are expected to provide valuable insight into the mechanisms of cell–cell communication in plants.

METHODS

Plant Material

The *Brassica oleracea* var *acephala* S_3 homozygous line has been described by Delorme et al. (1995).

cDNA Cloning and DNA Sequencing

Poly(A)$^+$ RNA, extracted from leaves of field-cultivated *B. oleracea* plants, was reverse transcribed, and the resulting cDNA was cloned into λ ZapII (Stratagene, La Jolla, CA). A polymerase chain reaction probe was generated by amplification of genomic DNA sequences of the S_3 homozygous *B. o. acephala* line, with two oligonucleotides corresponding to conserved regions of SLG_{29} (5′-CAGAGATGA-AACTGGGTTACGACC-3′ and 5′-AAATCACACAACCCGTCCC-3′). Plaque-forming units (500,000) of the primary library were screened with this probe, and hybridizing clones were plaque purified. The *SFR2* gene was isolated from a genomic library constructed with DNA of the same line (Delorme et al., 1995). Sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (1977), using the Sequenase system (version 2.0; U.S. Biochemical) and custom-synthesized oligonucleotides. Sequence data were analyzed with Lasergene sequence analysis software (DNASTAR, London, UK). The transcription start site of *SFR2* was determined by RNase protection mapping (Guardian RNase protection assay kit; Clontech, Palo Alto, CA).

Wounding and Infiltration of Bacteria and Inducers into *B. oleracea* Leaves

For the following experiments, *B. oleracea* plants were grown in a growth chamber at 22°C with a 16-hr-light period. Each experiment was repeated at least twice. For the wounding experiments, fully expanded leaves were wounded by rubbing the undersides with abrasive paper. Whole leaves were harvested for RNA extraction. In the bacterial infections, one strain of *Escherichia coli* (DH5α) and two strains of *Xanthomonas campestris* pv *campestris* were used for infections: one wild type (8004) and the other carrying a Tn*5* transposon insertion in the *hrp* gene cluster (8004::A2), which results in an *hrp* phenotype (Arlat et al., 1991). Pathogenicity tests were performed by infecting leaves in which a small notch had been cut at the end of a vein (Gough et al., 1988). With the *X. c. campestris* wild-type strain, symptoms appeared after 5 days and later spread to the rest of the leaf. No symptoms were observed after infection with the *X. c. campestris* 8004::A2 or *E. coli* DH5α. The effect of bacterial strains on *SFR2* expression was assessed by infiltrating (with a syringe) bacterial suspensions of 10^7, 10^8, or 10^9 cells mL^{-1} into an area (~2 to 3 cm^2) of the leaf through a small hole made with a needle. The infiltrated area was marked with a pen, and only this part of the leaf was harvested for RNA extraction.

Analysis of the growth of the two strains in the infiltrated area over a 4-day period showed that although the pathogenic strain 8004 had multiplied by a factor of 10^3, strain 8004::A2, which produced no visible symptoms, had multiplied only 10-fold. Salicylic acid (SA), SA analogs, abscisic acid (ABA), and methyl jasmonate (MeJA) were purchased from Sigma or Aldrich and infiltrated into leaves by the same procedure used for the bacterial suspensions. Control infiltrations were performed with the carrying solution minus the chemical inducer. Carrying solutions were 5 mM phosphate buffer, pH 7, for SA and SA analogs, 5 mM phosphate buffer, pH 7, 0.053% ethanol for ABA, and water for MeJA.

RNA Gel Blot Analysis

RNA was extracted from a range of tissues. The developmental stage of anthers was determined by fluorescence microscopy observation of 4′,6-diamidino-2-phenylindole–stained microspores and approximately correlated with bud length. Buds of <3 mm contained tetrads of microspores, 5-mm buds contained free uninucleate microspores, 5- to 7-mm buds contained both uninucleate and binucleate microspores, 8-mm buds contained binucleate microspores, 9- to 10-mm buds contained trinucleate microspores, and open flowers contained mature pollen grains. Total RNA was extracted by the method of Jackson and Larkins (1976). Total RNA (25 μg per lane) was separated on formaldehyde gels and stained with ethidium bromide to ensure that equal amounts of RNA had been loaded. The RNA was then transferred to nylon filters for hybridization. Equal transfer was controlled by visualizing RNA with ethidium bromide. DNA probes were prepared by using a random priming DNA labeling kit (Boehringer Mannheim). Filters were prehybridized and hybridized at 42°C in 50% formamide, 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, and herring sperm DNA (100 μg mL^{-1}). Filters were washed twice for 20 min in 0.1 × SSC and 0.1% SDS at 50°C. Autoradiographs were scanned for densitometry with an Arcus scanner (Agfa, St. Quentin-en-Yvelines, France), and the data were analyzed with Scan Analysis software (Biosoft, Cambridge, UK).

Construction of an *SFR2::uidA* Reporter Gene Fusion, Plant Transformation, and Analysis of β-Glucuronidase Activity

A 1520-kb DNA fragment containing the *SFR2* promoter region was generated by polymerase chain reaction amplification with two specific oligonucleotides: 5′-GGTGTCGACTTGTGTTCTTGTTTTTTC-3′ and 5′-GTCAAGCTTACTACCACCCGACC-3′. Restriction sites (SalI and HindIII) incorporated into the ends of the oligonucleotide sequences were used to insert the *SFR2* promoter upstream of the *uidA* reporter gene in the binary vector pBI101 (Jefferson et al., 1987), and the construct was transferred into *Agrobacterium tumefaciens* LBA4404. Tobacco plants (*Nicotiana tabacum* cv Xanthi) were transformed by the leaf disc method (Horsch et al., 1985), and transformants were selected on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing kanamycin (200 μg mL^{-1}) and cefotaxime (350 μg mL^{-1}). Transgenic plants (T_1) were self-fertilized, and seeds (T_2) were collected and sown on MS medium containing kanamycin (400 μg mL^{-1}). Seedlings were grown in vitro on MS medium for 4 weeks and then transferred to soil in a growth chamber at 25°C with a 16-hr-light period. The infection experiments were performed on 8-week-old kanamycin-resistant T_2 plants, with two plants per experimental condition. The bacterial suspensions were infiltrated at 10^8 cells mL^{-1} into tobacco leaves by the syringe infiltration procedure (Pontier et al., 1994). Three independent transformants were analyzed, and a similar pattern of expression was observed for all three transformants. All of the *Ralstonia solanacearum* strains were provided by C. Boucher (INRA-CNRS, Toulouse, France; Boucher et al., 1985) and have been described previously (Pontier et al., 1994). β-Glucuronidase (GUS) activity was assayed fluorometrically or histochemically on fresh tissue by using either 4-methylumbelliferyl β-D-glucuronide (Sigma) or X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Clontech), respectively, as the substrate (Jefferson et al., 1987). All experiments were repeated at least twice.

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