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(54) Title: NOVEL PROMOTERS

(57) Abstract: A promoter sequence associated with the tonoplast intrinsic protein *FavRB7* gene, or a variant or derivative thereof, allows tissue specific expression in the Rosaceae and constitutive levels of expression in other plants at levels similar to those observed with CaMV35S.

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NOVEL PROMOTERS

The present invention relates to promoters derived from *Fragaria spp.*, and uses therefor.

The field of plant transformation remained largely unexplored until the late 1980's, but this changed with the discovery of the unique abilities of *Agrobacterium tumefaciens*. Large numbers of plant species have now been transformed in order to incorporate suitable characteristics or traits, varying from pest resistance to hormone production. An example of the former is the production of *Bt* toxin, while an example of the latter is heightened expression of ethylene in tomatoes.

Despite the success in being able to express virtually any desired protein in a plant cell, relatively few promoters have been employed. Indeed, the standard promoter is the cauliflower mosaic virus 35S RNA (CaMV35S) promoter, as this leads to high levels of constitutive expression, both aspects of which can be valuable properties.

However, it has been established that CaMV35S has a recombination "hotspot", leading to worries that unforeseen recombination events, especially with viruses, could lead to spread of the associated gene, even to humans. In addition, constitutive expression throughout the plant is no longer necessarily desirable, as the gene associated with the promoter will necessarily be expressed in the part of the plant to be consumed, when the plant is a food crop. The constitutive nature of CaMV35S promoter is such that high levels of the associated gene are expressed in all tissues of the transformed plant.

Tissue-specific transgene expression is, therefore, a desirable target. However, the problem lies with the reliability and predictability of tissue-specific promoters from one species being similarly tissue-specific in another species.

For example, haemoglobin genes are preferentially expressed in the roots of several plant species (Appleby, C.A. *et al.* (1988), Plant Cell Environ. *11*, 359-367). These genes have also been isolated from non-nodulating species, in addition to the nodulating legumes in which they are most commonly found. However, use of the haemoglobin *par* promoter in a heterologous host (tobacco) met with limited success. Although the promoter was intended to lead to expression only in the roots, expression was additionally seen in the

leaves of 46 out of 140 transgenic lines (Van de Hoven *et al.* (1994), Transgenic Res.3, 159-165).

Similar findings exist for other root-specific genes, and none has yet been reported that remains identically tissue-specific across all species.

The cultivated strawberry is a complex plant. The modern cultivated strawberry, *Fragaria ananassa*, is a hybrid octaploid resulting from a cross of two New World octaploid species (*F. chiloensis* and *F. virginiana*). Breeding programs for the cultivated strawberry have concentrated primarily on improvements to plant vigour, fruiting habit, fruit quality, winter hardiness, frost tolerance and, to some extent, developing pest and disease resistance. However, the majority of genes in the modern strawberry are derived from only seven nuclear, and ten cytoplasmic, sources (Hancock, J. F., *et al.* (2001), Hort. Science *36*, 221-225). This has resulted in a narrow germplasm base, and sources of resistance are highly restricted, owing both to the narrow germplasm base and to the alternative requirement for interploid crosses.

The strawberry is susceptible to a wide range of pests and diseases, including both insect and fungal diseases. The primary insect disease is that of vine weevil infection, the larvae of which feed on both the crowns and the roots of the strawberry, often leading to plant death. The roots are also subject to infection by a number of soil borne fungi, such as *Verticillium dahliae* and *Phytophthora fragariae*, which generally cause the plant to die.

Methyl bromide has been used on a large scale in order to kill the vine weevil, but has been identified as a significant ozone-depleting substance, and is generally being withdrawn from commercial use. Broad-spectrum insecticides and fungicides have limited efficacy, and can also encourage the development of resistant strains of pests.

WO98/31812 identifies promoters from strawberries which are tissue-specific to the receptacle.

US-A-6043010 also discloses tissue-specificity in receptacles of strawberries, while WO 01/51637 discloses the isolation of a promoter from the wild strawberry which is specific, once again, for the receptacle, WO 02/34894 relating to similar subject matter.

Thus, tissue-specific promoters so far identified in relation to strawberries are specific for the fruit. This is not a problem if the gene to be promoted is to enhance flavour, for example, but genes controlled by promoters specific for the receptacle will only be expressed in the fruit, and will be unable to affect the plant as a whole, especially outside of the fruiting season. In addition, genetically engineered expression in the fruit is less likely to meet with public acceptance.

Thus, there remains a requirement for a constitutive promoter for plants that has similar activity to that of CaMV35S, and which preferably is not associated with a virus.

There is also a desire for tissue-specific promoters in food crop plants.

A novel tonoplast intrinsic protein (TIP) gene homologue has now been identified in strawberry, which is expressed primarily in root tissue, and the promoter of which is useful in the expression of foreign genes.

Thus, in a first aspect, the present invention provides a promoter sequence associated with the tonoplast intrinsic protein gene homologue, FavRB7, from strawberry, or a variant or derivative thereof, especially as shown in SEQ. ID NO. 1.

In an alternative aspect, there is provided a promoter sequence as shown in SEQ. ID NO. 1, or a variant or derivative thereof.

The preferred promoters are associated with the tonoplast intrinsic protein gene homologue FavRB7 found in the strawberry, by which is meant that this gene has been found to be controlled by this promoter, and that variants and derivatives of this promoter sequence associated with FavRB7, preferably as found in nature, are included in the invention.

The preferred promoter of the present invention is designated SEQ ID NO. 1 in the accompanying Sequence Listing. The same sequence is illustrated in Figure 1, but has the ATG initiation codon.

It is preferred that the promoter is operably linked to an initiation codon, by which is meant that the promoter promotes transcription of a translatable sequence containing the codon as a translation initiator.

The promoters of the present invention are constitutive or tissue-specific, depending on the plant species in which they are used and/or the nature of the derivative of the promoter. In particular, when used in conjunction with *Rosaceae spp.*, and especially *Fragaria spp.*, the promoters of the invention are largely root-specific while, in other species, such as tobacco, the promoters of the invention tend to be constitutive.

Whether the promoters of the invention are constitutive or tissue-specific, they have advantages over the prior art. Where the promoters are constitutive, they are of plant origin and are not associated in any way with viruses, so that any threats associated specifically with viral promoters are avoided. Although such threats may not be as substantial as public opinion would suggest, the effect on sales can be very substantial. Moreover, the *FavRB7* gene promoter confers activity similar to that of the CaMV35S promoter when used in a heterologous environment, so that it may readily be used in the place of the CaMV35S promoter, where such is currently used or considered for use, for example.

Especially in the species Rosaceae, promoters of the present invention are generally root-specific. In fact, expression is confined mainly to the root, but some expression has been noted in the petiole. Virtually no expression is observed in the leaves, floral organs or fruit, so that any gene associated with the promoters of the present invention will not be expressed in the fruit, thereby minimising or obviating the risk of the consumer eating the expression product.

It will be appreciated that the term "tissue-specific", and associated terms, indicate that the promoter is active primarily in a given tissue, or range of tissues, and that it will be substantially dormant in at least one tissue type. For the purposes of the present invention, the term "dormant" is taken to mean that activity of the promoter, such as by detection of associated marker activity, is substantially undetectable in the tissue in which the promoter is dormant. For example, if the promoter is associated with the *gusA* marker, then the tissue in which the promoter is dormant will not appear to have any blue colour when subject to histological analysis.

The term "variants" includes all forms of the promoter found in nature within the genus *Rosaceae* and, particularly, within the *Fragariae.* Although this may encompass various deletions, insertions, inversions and replacements, variants are generally of

identical, or very nearly identical, length, and any difference is generally limited to replacement.

Variants preferably have an identity of at least 80% with SEQ ID NO 1. More preferably there is at least 90% identity, such as at least 95% or at least 98% identity.

"Identity" is a measure of the identity of nucleotide sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognised meaning and can be calculated using published techniques. See: *e.g.*: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed.: Academic Press, Now York, 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heinje, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

While there exists a number of methods to measure identity between two polynucleotide sequences, the term "identity" is well known to skilled artisans (Carillo, H., and Lipton, D., *SIAM* J Applied Math (1988) 48:1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Marlin J. Bishop, ed., Academic Press, San Diego, 1 994, and Carillo, H., and Lipton, D., *SIAM* J Applied Math (1988) 48:1073.

The present invention also extends to "derivatives" of the promoter of the *FavRB7* gene, and these include any modified version of the promoter, provided that the activity of the promoter is retained to the extent that it remains essentially root-specific in *Fragaria spp.* Derivatives include mutations such as deletions, insertions, inversions, replacements and truncations.

Truncated forms of the promoter should preferably be no less than 600 base pairs in length, and preferably between 1400 and 2500 base pairs. Where convenient, if there is no requirement for amending the promoter sequence, then it is generally preferred to use the full sequence of SEQ ID NO. 1, or preferably at least 50% of the 3' end, together with the

ATG codon, and more preferably 70% and more preferably 80% of the 3' end. Amendments may include incorporating splice sequences, for example.

The *FavRB7* gene promoter sequence was subject to a search for *cis*-acting regulatory elements using the PlantCARE database (www.oberon.rug.ac.be). This search identified only one regulatory motif associated with root-specific expression, an *as1* motif at position - 2271. However, several regulatory motifs linked to hormone response were identified; auxin response, an AuxRR-core element at - 215 and TGA elements at - 1975 and - 2271; an abscisic acid response, CE1 element, at - 198; ethylene response, ERE, elements at – 542, - 935, 1184 and - 2113 and gibberellin response, P-box and TATC box, elements at - 785, - 1868, - 1904 and - 503.

Several regulatory motifs associated with environmental stresses were also identified; drought inducibility, MBS, elements were found at - 68, - 2069 and - 2185; a low temperature response, LTR, element at - 1557; heat responsive elements, HSE at - 274, - 893, - 1009, - 1107, -1145 and - 2213; wound response, WUN, motifs were found at - 38, - 544, - 815, - 1186, - 1343, - 1657, - 1734, - 2115, - 2281 and - 2617 and an anoxic specific inducible, GC-motif was found at - 126.

It will be appreciated that motifs may be added to the sequence, where they are not already present, or where it is desired to supplement the existing motif, or motifs may be deleted. Any one of the motifs listed above, or a combination thereof, may be deleted from the promoter sequence, in order to disable variable expression. For example, deleting one or more of the motifs corresponding to hormone responses can stabilise resulting expression at consistent levels, unaffected by growing conditions.

Several regions of repetitive sequence were identified in the *FavRB7* promoter (Figure 1), the longest of which is a direct repeat of 22/23 bases occurring at positions - 1798 / - 1819. A direct repeat of 13 bp occurs at - 718 / - 772 and four 11 bp direct repeats were also identified. A 15 bp dyad repeat was identified at - 1883 / - 1416 and a 12 bp dyad repeat at – 1350 / – 942. Inverted repeats of 12 and 11 bp were found at – 2033 / - 537 and – 2429 / - 1817.

Figure 1 shows the nucleotide sequence of the *FavRB7* gene promoter. Nucleotide positions are given distal to the proposed ATG *FavRB7* gene initiation codon. Putative

regulatory motifs are indicated in bold:- As1- root specificity, AuxRR - auxin response, TGA – auxin response, CE1 - abscisic acid response, ERE - ethylene response, P-box and TATC boxes – gibberellin response, MBS - drought inducibility, LTR - low temperature response, HSE - heat responsive elements, WUN- wound response and GC-motif - anoxic inducible. Regions of repetitive sequence identified are underlined and highlighted in grey, the type of repeat is indicated and letters indicate pairs of repetitive sequence.

There is no particular restriction on the nature of the gene or genes that may be operably associated with the promoter of the present invention, and neither is there any restriction on the number of occasions the promoter of the invention may be used in any one genome. For example, the promoter may be associated with a *Bt* toxin gene, together with any other appropriate sequences, such as a termination sequence, and the resulting operon may be incorporated once or a number of times into the genome of a desired plant. The promoter may also be associated with another gene, such as a gene involved in the synthesis of ethylene, and this may also be incorporated once or a number of times into the genome of the same or another plant. The operon usefully further comprises a termination sequence and any other expression sequences that may be desired.

It will be appreciated that the promoter and any associated sequence may be incorporated at random, on a vector, or by targeted transformation techniques, all of which are well known in the art.

Exemplary genes for use with the present invention in order to confer resistance against some of the insect pests of strawberry, for example, include the proteinase inhibitors, *Bacillus thuringiensis* (*Bt*) toxins and plant lectins. Proteinase inhibitors generally form complexes with proteinases, thereby inhibiting enzyme activity and, thus, disrupting digestion in insects that consume these compounds. *Bt* toxin gene products are δ-protoxins that form an active toxin during digestion by insects that consume the δ-protoxin. Plant lectins act by binding to carbohydrates and also have a disruptive effect on insect metabolism.

Suitable genes for conferring resistance against fungal pathogens of strawberry, for example, are the endochitinases, which hydrolyse the chitin component of fungal cell walls; various antimicrobial peptides which have exhibited antimicrobial activity against bacteria and fungi when expressed in transgenic plants (Park, *et al.* (2000), Plant Mol.

Biol. *44*, 187-197; DeGray, *et* al., (2001), Plant Physiol. *127*, 852-862); the beta-1-3-glucanase protein, which has been shown to confer a degree of resistance to several fungal pathogens in tobacco and wheat; the ribosome-inactivating proteins (RIPs), which have displayed varying antifungal and antiviral activities (Bieri, S., *et* al., (2000), Theor. Appl. Genet. *100*, 755-763; Nielsen and Boston, (2001) Annu. Rev. Plant Physiol. Plant Molec. Biol. 52, 785-816; Park *et al.*, (2002) Planta 216, 227-234; Parkash *et al.*, (2002) Peptides 23, 1019-1024). These last often serve as plant defence proteins, and specifically inhibit protein synthesis through the depurination of the conserved alpha-sarcin loop of large ribosomal RNAs (Park *et al.*, *supra*).

Sequence homology analysis between the *FavRB7* and *TobRB7* (EMBL: S45406) promoters revealed 23% overall homology between the two sequences. Repetitive sequence elements designated B and C boxes in the *TobRB7* promoter were not present in the *FavRB7* promoter. However, the sequence motif, CGAGCTCGATA (SEQ ID NO. 10), which forms the consensus of the *TobRB7* promoter A box, was present several times in the *FavRB7* promoter (Figure 2).

Figure 2 shows sequence positions distal to the *FavRB7* gene initiation codon for sequence motifs identified in the *FavRB7* promoter homologous to the A box motifs identified in the *TobRB7* promoter. Regions of homology are indicated by black or grey shading; regions of divergence are highlighted in white. The *TobRB7* promoter A box consensus sequence is presented below the *FavRB7* sequence motifs in bold and underlined.

The invention provides an operon comprising at least a promoter as defined, in operable association with a gene, preferably a gene having an expression product.

In general, it has been established that there is no particular advantage to incorporating the operon any more than once into any given genome and, indeed, there may be disadvantages associated with multiple occurrences where the primary insertion event is random.

It is generally preferred to associate the promoter of the present invention with a marker therefor, in order to be able to establish whether a particular cell line has been transformed, and to follow the efficiency of transformation. Suitable markers may include

resistance markers, such as kanamycin, gentamycin, tetracycline, streptomycin and spectinomycin, although there is popular antipathy to such markers, owing to the possibility of spreading resistance to other species. A particularly useful marker is the *gusA* marker referred to above. The product of this gene is β-glucuronidase which, when incubated with the substrate 5-bromo-4-chloro-3-indolyl-1-glucuronide, produces a blue stain visible to the naked eye. The lack of detectable levels of β-glucuronidase in plants and the wide availability of spectrophotometric substrates for the enzyme, makes the *gus*A system particularly suitable as a marker.

Other selectable markers, such as other antibiotic resistance markers, non-antibiotitc resistance markers, including phosphomannose/xylose isomerase, and other scorable markers, such as green fluorescent protein (GFP), and luciferase may also be used.

The present invention also extends to vectors, such as plasmids, comprising a promoter of the present invention, especially where the promoter is associated with a gene to be expressed in a plant.

The present invention further extends to methods for incorporating promoters of the present invention into plants, especially where the promoter is associated with a gene to be expressed. Such methods may include microinjection, electroporation, PEG-transformation, transformation with a vector, and biolistics, such as exemplified by Sanford, *et al* (Method Enzymol. (1993), *217*, 483-509).

The present invention envisages plants, or reproductive material thereof, comprising a promoter as defined above in expressible form. Thus, it is preferred that the promoter be in operable connection with at least one gene. The promoter is preferably incorporated in to the genome, although it may be present in a vector.

It will be appreciated that reference to a nucleotide sequence of the invention can generally be taken to include reference to a sequence comprising at least a promoter of the invention. Preferred sequences are DNA.

The present invention also envisages a plant expressing at least one transgene in at least one tissue thereof at at least one stage in the lifecycle thereof, wherein the transgene is under the control of a promoter as defined above. In this respect, a transgene may be

either homologous or heterologous, although the latter is preferred, and is a gene not normally expressed in association with a promoter of the invention. For example, a gene normally expressed only in the fruit may be expressed in the root, but it is generally preferred that a heterologous gene be expressed constitutively, or in a tissue specific manner, in the plant.

The present invention will now be illustrated by way of the following, non-limiting Example.

EXAMPLE 1

Construction of the binary vector, pSCVFavRB7

The binary vector pSCV1.6 (Figure 3) harbours the *gusA* reporter gene driven by the CaMV 35S promoter next to the left T-DNA border and the neomycin phosphotransferase (*nptII*) gene also driven by the CaMV 35S promoter, next to the right T-DNA border. This vector was used to confer constitutive *gusA* expression, to serve as a comparison to the *FavRB7* gene promoter in these investigations.

To create a vector comparable to pSCV1.6, but with the *gusA* gene under control of the *FavRB7* promoter, the CaMV 35S RNA promoter controlling the *gusA* gene in pSCV1.6 was removed and replaced with the 2843 bp *FavRB7* gene promoter isolated from strawberry, creating the vector pSCVFavRB7.

Primers, FavRB7SS and FavRB7N (SEQ ID NO's 8 and 9, respectively), were designed to incorporate *Sph* I and *Sma* I sites at the 5' end of the promoter and an *Nco* I site across the initiation codon of the *FavRB7* gene to facilitate cloning. PCR was carried out using the high fidelity proof reading enzyme *Pfx* and pFavRB72.8 as template. A 2860 bp amplification product incorporating the *FavRB7* promoter sequence and *FavRB7* gene 5' untranslated region up to the ATG initiation codon was gel isolated and transformed into the vector pCR-Blunt-TOPOII. Plasmid DNA was prepared from transformed colonies and sequenced to confirm the correct addition of restriction sites. The authenticated plasmid was named **p*FavRB7P*.**

Figure 3 shows the binary vector pSCV1.6. Left (LB) and right (RB) T-DNA borders are indicated. OD indicates an overdrive T-DNA transfer enhancer. Arrows indicate the orientation of promoters and genes. Key restriction endonuclease sites are indicated and their positions noted.

The plasmid pFavRB7P was digested with *Sph* I and *Nco* I and the resulting 2856 bp restriction fragment ligated into *Sph* I and *Nco* I cut, dephosphorylated pGUS358-S. The plasmid pGUS358-S (EMBL: U02441) harbours the *gusA* gene with an *Nco* I site across the initiation codon. Correct linkage of the *FavRB7* gene promoter to the *gusA* gene in pGUS358-S was confirmed by sequencing and the authenticated plasmid named **pGFavRB7P**.

Plasmid pGFavRB7P was digested with *Sma* I and *Sna* BI to release a 3228 bp fragment representing the *FavRB7* promoter and the first 385 bp of the *gusA* gene. This fragment was then ligated into *Sma* I and *Sna* BI digested, dephosphorylated pSCV1.6 replacing the CaMV 35S promoter and the first 385 bp of the *gusA* gene. Correct linkage of the components was confirmed by sequencing and the authenticated binary vector containing the *FavRB7* promoter linked to the *gusA* gene named **pSCVFavRB7** (Figure 4).

Both pSCV1.6 and pSCVFavRB7 were transformed into electro-competent cells of *Agrobacterium tumefaciens* strain EHA101 prior to use in nuclear transformation experiments.

***FavRB7* promoter activity in strawberry and tobacco transgenics**

a) *Agrobacterium*-mediated nuclear transformation

Strawberry, cv. Calypso, and tobacco cvs Ottawa and Petit Havana were transformed using the binary vectors pSCV1.6 and pSCVFavRB7 in *Agrobacterium tumefaciens* strain EHA101. Shoots regenerating from transformation experiments for both strawberry and tobacco were maintained on selective medium for a minimum of 12 weeks prior to weaning.

Figure 4 shows the binary vector pSCVFavRB7. The *FavRB7* gene promoter replaces the CaMV 35S promoter driving *gusA* in pSCV1.6 to create pSCVFavRB7. Left (LB) and right (RB) borders are indicated. OD indicates an overdrive T-DNA transfer enhancer. Arrows indicate the orientation of promoters and genes. Key restriction endonuclease sites are indicated and their positions noted.

Two replicates of each putative transgenic strawberry line were weaned and transferred to a controlled environment cabinet. After six weeks under standard growth conditions the plants were subject to a 14 d chilling treatment of 10°C day and night to induce flowering. After a further six weeks under standard growth conditions the plants were transferred to the glasshouse under natural heat and light. All strawberry lines produced displayed the phenotype typical of Calypso. Runners generated from the R0 lines were rooted and then detached to produce R1 lines. All R1 lines produced displayed the typical phenotype.

Tobacco plants were weaned and transferred to the glasshouse. All of the tobacco lines produced displayed the typical phenotype for *Nicotiana tabacum*.

Primers FavPGUS1 and FavPGUS2 (SEQ ID NO's 2 and 3, respectively) were designed to anneal in the *FavRB7* gene promoter and the *gusA* gene respectively, and were used in PCR to confirm the presence of this region in pSCVFavRB7 transgenic lines of both strawberry and tobacco. Primers GUS27 and GUS392 (SEQ ID NO's 6 and 7, respectively) were used to confirm the presence of the *gusA* gene in pSCV1.6 transgenic lines.

Thirty-two strawberry lines containing pSCVFavRB7 and one line containing pSCV1.6 T-DNA were generated. In tobacco, eight Ottawa lines containing pSCVFavRB7 and two lines containing pSCV1.6 T-DNA were generated. Eleven Petit Havana lines containing pSCVFavRB7 and two lines containing pSCV1.6 were also generated. PCR was used to detect the *FavRB7* gene promoter and *gusA* gene in these lines. Strawberry lines 50, 59a, 59b and 60, and tobacco lines 35 and 68 transformed with pSCVFavRB7 were additionally generated, however the *FavRB7* gene promoter: *gusA* region was not detected in these lines.

Southern analysis of transgenic strawberry

Southern analysis was carried out on 36 putative pSCVFavRB7 lines and one putative pSCV1.6 line of strawberry. The transgenic tobacco lines generated were not subject to Southern analysis due to time constraints.

To establish site integration number of the *gusA* and *nptII* genes and to confirm the presence of the *FavRB7* gene promoter:*gusA* gene region in transgenic strawberry lines, genomic DNA was digested with *Kpn* I and *Sma* I. Digoxigenin-II-dUTP (DIG) labelled *nptII* and *gusA* probes were synthesised using the primers NPTIIA and NPTIIB and GUS27 and GU392, respectively, using pSCV1.6 plasmid DNA as template in PCR. These probes were then used to evaluate the number of transgene integration sites.

Southern analysis of *Kpn* I digested genomic DNA revealed the number of integration sites for the *nptII* gene, which was confirmed through Southern using *Sma* I digested genomic DNA. Southern analysis of *Sma* I digested genomic DNA revealed the number of integration sites for the *gusA* gene.

The presence of a second *Kpn* I site within the T-DNA (Figure 4) meant that a 4.6 kb restriction fragment representing the *FavRB7* gene promoter:*gusA* gene region should be detected in all pSCVFavRB7 transgenic lines using the *gusA* probe. Identification of fragment greater than or less then 4.6 kb would imply that some manner of deletion or rearrangement has occurred within this region to remove a *Kpn* I site at either the 5' of 3' end of this region. In both cases it is important to note that the integrity of the promoter driving *gusA* cannot be guaranteed. Evidence of such alterations were observed in lines 41, 53a, 53b, 61, 67, 72 and 78.

Southern analysis established the number of integration sites for both *nptII* and *gusA* genes in the transgenic lines generated (Table 1). In the majority of the transgenic lines integration of both the *nptII* and *gusA* genes was found to have occurred at a single site, 20/34 and 27/34, respectively. Six lines (41, 53a, 53b, 67, 78 and 102) were found to contain multiple insertions of both genes. In four lines (41, 53a, 53b and 67) the integrity of the *FavRB7* gene promoter:*gusA* gene region could not be confirmed at all integration sites. Lines 58a and 58b were found to be clonal, having arisen from a single transformation event, and so only line 58a was subject to further analysis. Lines 50, 59a,

59b and 60 were found to contain neither the *nptII* or *gusA* gene, which supported the PCR observations for these lines. These lines were not subject to further analysis.

Line	*gusA*	*nptII*	Line	*gusA*	*nptII*
-	0	0	**55**	1	2
1	1	2	**58a**	1	2
5b	1	2	**58b**	1	2
5c	1	1	**59a**	0	0
6	1	1	**59b**	0	0
13	1	1	**60**	0	0
14	1	1	**61***	1	1
37	1	1	**64**	1	1
38	1	1	**65**	1	1
39	1	1	**66**	1	1
41*	2	7	**67***	2	1
42	1	2	**71**	1	1
43	1	1	**72***	2	2
44	1	1	**75**	1	1
46	1	1	**76**	1	1
48	1	2	**77**	1	1
49	1	1	**78***	2	2
50	0	0	**102**	2	2
53a*	4	5	+	1	2
53b*	3	4			

Table 1 Number of integration sites observed for *gusA* and *nptII* in pSCVFavRB7 strawberry lines

The number of integration sites of each transgene in each pSCVFavRB7 strawberry line is indicated. Non-transformed control (-) and pSCV1.6 (+) lines are included. An asterisk denotes those lines highlighted in Figure 4.8, which may contain deletions or rearrangements within the *FavRB7* gene promoter:*gusA* gene region.

Histochemical analysis of transgenic strawberry

A β-glucuronidase (X-Gluc) histochemical assay was carried out to assess expression of the *gusA* gene in a range of tissue types in the pSCVFavRB7 transgenic strawberry lines. Leaf, petiole and root samples were collected from all glasshouse grown lines. Not all lines flowered within the study period, however where possible floral organs (whole buds and open flowers) were also collected. All samples were stored at -80°C prior to analysis.

Histochemical analysis was carried out. Blue staining was observed in the roots of all pSCVFavRB7 transgenic lines, at similar levels to that observed in the CaMV 35S line. No blue staining was observed for the *FavRB7* gene promoter lines in the leaves or floral organs, whereas intense blue staining was observed in the CaMV 35S line. Light blue staining was observed in the petioles of several of the *FavRB7* gene promoter lines, although not to the intensity of the CaMV 35S line, suggesting that *gusA* is also expressed to a lesser degree in the petioles of some *FavRB7* gene promoter lines. This suggests that the *FavRB7* gene promoter confers expression of *gusA* preferentially in the roots of strawberry.

Further samples of each tissue type were taken from non-transformed control, the CaMV 35S line and pSCVFavRB7 lines 37 and 53b and subject to histochemical assay. These samples were then fixed in 10% formalin prior to histological study. To obtain detailed visualisation of *gusA* activity within the various tissue types, 10 μm sections were taken from the various tissues and photographed under magnification using the Zeiss axiophot microscope and a Nikon coolpix digital camera.

Intense staining as a result of *gusA* activity was observed in all tissues of the CaMV 35S line and was non-detectable in the non-transformed control line. Intense staining was only observed in the root tissues of the pSCVFavRB7 lines and no staining was observed in the leaf, floral organs or fruit of the pSCVFavRB7 lines.

However, slight staining was observed in the petioles. Microscopy revealed that *gusA* activity in the roots of the pSCVFavRB7 lines was highest in the central vascular region of the root and in the tip of the lateral root cap. Intense staining was often observed in the root tips, however it was thought that this may be a result of older root

tissues being more highly suberised and less permeable to the β-glucuronidase substrate used in the histochemical assay rather than lack of activity in the older tissues.

To establish if the expression pattern conferred by the *FavRB7* gene promoter was maintained through vegative propagation in strawberry histochemical analysis was also carried out on a population of eight R1 lines, propagated from stolons of R0 pSCVFavRB7 lines. In all of the lines examined, the spatial expression pattern observed in the R0 lines of strong blue staining in the roots, weak staining in some petioles and no staining in the floral organs or leaves, was maintained in the R1 lines. This suggests that the transgene expression pattern conferred by the *FavRB7* gene promoter is maintained through vegetative propagation.

The pSCVFavRB7 transgenic tobacco lines were also subject to β-glucuronidase (X-Gluc) histochemical assay to assess the activity of the *FavRB7* gene promoter in a heterologous system. Tobacco tissue samples were collected from the leaf at the fourth internode, the petiole of this leaf, stem tissue above the fourth internode, young root tissue and floral organs. Comparable levels of blue staining were observed in all tissues for lines transformed with pSCV1.6 and pSCVFavRB7. Although not quantitative, the histochemical assay showed that *gusA* expression levels were uniformly high in all tissues and not preferentially expressed in the roots of tobacco. This result clearly shows that *FavRB7* gene promoter activity differs significantly in tobacco compared to strawberry, behaving constitutively in the heterologous system.

GUS fluorometric analysis of transgenic strawberry

Fluorometric analysis was carried out to quantitatively assess the expression pattern of the *gusA* gene under control of the *FavRB7* gene promoter in strawberry. Leaf, petiole and root samples were collected from one pSCV1.6 and 32 pSCVFavRB7 lines of glasshouse grown transgenic strawberry. Not all clones flowered during the study period and no mature fruit was available for fluorometric analysis. However, floral organs, whole buds and flowers were collected from those clones producing flowers over a period of several weeks and stored at – 80°C.

To account for variation within the lines, samples were taken from 2 replicates of each line and a minimum of 6 replicates of each tissue type collected. For each line, leaves were excised using a 1 cm cork borer and petiole and root tissues were excised in sections of ~0.25 cm and tissues were flash frozen in liquid nitrogen. Floral organs stored at - 80°C were pooled prior to analysis. An aliquot of approximately 100 mg of each tissue type was taken for fluorometric assay.

Fluorescence was quantified using a FLUOstar Galaxy multi well plate reader with the excitation filter set at 360 nm and the emission filter set at 460 nm. Each fluorescence reading was taken a minimum of four times and the average value calculated. Specific activity of *gusA* was quantified in terms of pmol 4-MU produced per min per mg total protein. Total protein was determined using the BioRad assay using the FLUOstar Galaxy multi well plate reader with the absorbance filter set at 595 nm. Each protein assay was read a minimum of four times and the average value calculated.

The fluorometric data generated from the 32 pSCVFavRB7 lines analysed revealed an average specific activity for *gusA* of 36,780 pmol 4-MU/min/mg in the roots and a comparable value of 40,482 pmol 4-MU/min/mg in the roots of the CaMV 35S line. By contrast, average *gusA* specific activities in the petioles, leaves and floral organs in the pSCVFavRB7 lines were found to be greatly reduced compared to the *gusA* expression in the roots. Average *gusA* specific activities for petiole, leaf and floral organs were found to be 7,417, 858 and 419 pmol 4-MU/min/mg respectively. For the same tissues CaMV 35S values were 44,908, 132,387 and 75,953 respectively. In pSCVFavRB7 lines the median *gusA* specific activity ratio between root: petiole was found to be 1: 0.3, root: leaf 1: 0.04 and root: floral organ 1: 0.009. These data corroborate the observations made using the histochemical assay that the *FavRB7* gene promoter confers expression of the *gusA* gene in a near root-specific manner.

Further examination of the fluorometric data revealed that the majority (25/32) of *gusA* specific activities in roots were over 10000 pmol 4-MU/min/mg for the pSCVFavRB7 lines. The lowest and highest values observed in root tissue were 3,518 and 154,500 pmol 4-MU/min/mg, respectively. By contrast the majority (25/32) of *gusA* specific activities in leaves were beneath 900 pmol 4-MU/min/mg. The lowest value observed for leaf tissue was 100 pmol 4-MU/min/mg. The majority (23/32) of

gusA specific activities observed in petioles were beneath 10,000 pmol 4-MU/min/mg. However, lines 1 and 55 both showed specific activities of over 30,000 pmol 4-MU/min/mg, which although lower than the root specific activities observed in these lines, is comparable with root specific activities for *gusA* observed in other lines. Only 17 lines contributed to the data generated for *gusA* specific activity in pSCVFavRB7 floral organs, of these lines the majority (13/17) exhibited specific activities beneath 160 pmol 4-MU/min/mg.

No significant correlation could be made between the number of copies of the *gusA* gene present in each line and the expression levels observed, however the majority (4/6) of those lines containing two or more copies of the *gusA* gene ranked in the bottom 30 % for *gusA* root expression levels. By contrast the line shown to have the second highest level of *gusA* expression in the roots (53a) contains four copies of the *gusA* gene and was implicated as containing possible rearrangements in the *FavRB7* gene promoter: *gusA* gene region. Anomalous levels of *gusA* gene expression were noted in several of the other lines suspected to contain such rearrangements. Line 53b consistently displayed very low *gusA* expression levels in all tissue types other than roots, and lines 61 and 78 displayed the highest levels of *gusA* expression observed in floral organs and leaf tissues, respectively.

Reverse transcriptase PCR analysis of transgenic strawberry

Expression of the *nptII* and *gusA* genes were studied at the molecular level in both pSCVFavRB7 and pSCV1.6 lines of transgenic strawberry using reverse transcriptase PCR (RT-PCR). Messenger RNA extraction, cDNA synthesis and PCR were carried out. Primers NptIIA, NptIIB (SEQ ID NO's 4 and 5, respectively), GUS27 and GUS392 were used in a multiplexed RT-PCR reaction to detect expression of *nptII* and *gusA* transgenes in the root, leaf, petiole, floral organs and fruit. Lines 6, 14, 37, 46 and 55 were selected from the pSCVFavRB7 strawberry transgenics because fluorometric analysis had shown these lines to represent a range of *gusA* expression levels and samples were available for all of the tissue types examined in the fluorometric study. Lines 6, 46 and 55 also produced fruit during the study period.

cDNA was synthesised from fruit tissues for these lines as well as the CaMV 35S constitutive control line.

Amplification fragments corresponding to the *nptII* and *gusA* genes were generated from all tissue types for the CaMV 35S line using RT-PCR. This confirmed that the assay was sensitive enough to detect the transgenes being expressed under control of the CaMV 35S promoter. Expression of the *nptII* gene under control of the CaMV 35S promoter was detected in all of the pSCVFavRB7 transgenic lines. The amplification fragment generated for the *gusA* gene under control of the *FavRB7* promoter was noticeably less intense than the *npt*II fragment generated in the pSCVFavRB7 lines. Only in the root tissues of line 55 was a *gusA* amplification fragment generated at similar levels to that of *nptII*. Although not quantitative this assay confirmed *gusA* activity conferred by the *FavRB7* promoter at the molecular level as was previously revealed through histochemical and fluorometric assay.

The *FavRB7* gene promoter isolated from strawberry was shown to act in a strongly root preferential manner in strawberry. Histochemical, fluorometric and molecular analyses revealed that its activity, as measured by activity of the expressed product of the *gusA* gene linked to the promoter, was low in tissues other than the root. In root however, *FavRB7* promoter activity was high and comparable to that of the CaMV 35S promoter.

Histochemical analysis showed that the *FavRB7* gene promoter imparts a very different spatial gene expression pattern in tobacco to that observed in strawberry. In tobacco the *FavRB7* gene promoter was found to act in a strong constitutive manner and accordingly may provide a useful alternative the CaMV 35S promoter in heterologous systems.

Claims:

1. A nucleic acid sequence comprising a promoter sequence as shown in SEQ. ID NO. 1, or a variant or derivative thereof.

2. A nucleic acid sequence according to claim 1, wherein the promoter is as found in association with a tonoplast intrinsic protein gene in the strawberry.

3. A nucleic acid sequence according to claim 1 or 2, wherein the promoter is operably linked to an ATG initiation codon.

4. A nucleic acid sequence according to any preceding claim, wherein the promoter is operably linked with a marker therefor.

5. A nucleic acid sequence according to claim 4, wherein the marker is *gusA*.

6. A nucleic acid sequence according to claim 4, wherein the marker is selected from: antibiotic resistance markers, non-antibiotitc resistance markers, and scorable markers.

7. A nucleic acid sequence according to any preceding claim, wherein the the promoter is a variant having an identity of at least 80% with SEQ ID NO 1.

8. A nucleic acid sequence according to claim 7, wherein the identity is at least 90%.

9. A nucleic acid sequence according to claim 8, wherein the identity is at least 95%.

10. A nucleic acid sequence according to claim 9, wherein the identity is at least 98%.

11. A nucleic acid sequence according to any preceding claim, wherein the promoter is a truncated form of SEQ ID NO. 1.

12. A nucleic acid sequence according to claim 11, wherein the truncated promoter is no less than 600 base pairs in length.

13. A nucleic acid sequence according to claim 11 or 12, wherein the length of the truncated promoter is between 1400 and 2500 nucleotides.

14. A nucleic acid sequence according to any preceding claim, wherein the promoter comprises at least 50% of the 3' end of SEQ ID NO. 1.

15. A nucleic acid sequence according to claim 14, wherein the promoter comprises at least 70% of the 3' end of SEQ ID NO. 1.

16. A nucleic acid sequence according to claim 14, wherein the promoter comprises at least 80% of the 3' end of SEQ ID NO. 1.

17. A nucleic acid sequence according to any preceding claim, wherein the promoter comprises the full sequence of SEQ ID NO. 1.

18. A nucleic acid sequence according to any preceding claim, further comprising one or more response motifs located within the promoter sequence.

19. A nucleic acid sequence according to any preceding claim, wherein one or more response motifs occurring within SEQ ID NO. 1 are deleted.

20. A nucleic acid sequence according to any preceding claim, wherein the promoter is operably linked with at least one gene encoding an expression product.

21. A nucleic acid sequence according to claim 20, wherein the gene encodes pest resistance.

22. A nucleic acid sequence according to claim 21, wherein the resistance is to a pathogen.

23. A nucleic acid sequence according to claim 21 or 22, wherein the resistance is to a fungus.

24. A nucleic acid sequence according to claim 20 or 21, wherein the expression product is selected from: proteinase inhibitors, *Bacillus thuringiensis* (*Bt*) toxins and plant lectins, and combinations thereof.

25. A nucleic acid sequence according to claim 20 or 21, wherein the expression product is selected from: endochitinases; antimicrobial peptides; beta-1-3-glucanase protein; ribosome-inactivating proteins, and combinations thereof.

26. A sequence according to any preceding claim, which is DNA.

27. A plant, or reproductive material thereof, comprising a nucleic acid sequence according to any preceding claim in expressible form.

28. A plant, or reproductive material thereof, according to claim 27, wherein the promoter is constitutive.

29. A plant, or reproductive material thereof, according to claim 27, wherein the promoter is tissue-specific.

30. A plant, or reproductive material thereof, according to any of claims 27 to 29, which is *Rosaceae spp.*

31. A plant, or reproductive material thereof, according to claim 30, which is *Fragaria spp.*

32. A plant, or reproductive material thereof, according to any of claims 27 to 31, wherein the promoter is root-specific.

33. A plant, or reproductive material thereof, according to any of claims 27 to 29, that is tobacco.

34. A vector comprising a nucleic acid sequence according to any of claims 1 to 26.

35. A vector according to claim 34 that is an expression vector.

36. A plant expressing at least one transgene in at least one tissue thereof at at least one stage in the lifecycle thereof, wherein the transgene is under the control of a promoter as defined in any of claims 1 to 26.

-2843 CGACGGCCCGGGCTGGTCTGCGAGTGACT

-2814 TTTATACCTCAAATCGTGTACCTTGATATTAAGTTTTCTTGTTTCTGTTATTGCTCTCAT

-2754 CATATCCTTTCTGATATTGGTCTTGTGATTGTAAGGTTTCGTATCAGTGTTCTATACTTT

-2694 TTGGATATTAATGGTTTGTTTCATCTGCAAAGATCGATTGGTTTCTGGTGTTTTTTTTTA

-2634 AAGAGGAAGGGAATGGT**TCATTGCTT**AATTTTATGTTCGATGGCAGGTTAATGTACTTAC

WUN

-2574 TGTTTCCATGTTTGCTCTTTTGTTGATTTGAATACAATGGTGTCAATTGATGTTGATTGT

-2514 AGCTAGTAAACTTGAGTGCTTACATGTACTACGTTTCGGTGTGTTCTTGTTGGTTTTGCT

-2454 TCCTAATGTGTGAGGTAAGTGTCTTGCCCAAAAAGAAAGAGATGAGGCTTTTAATTTATT

INVERTED REPEAT (b)

-2394 TGGATTGATCTTGTTATGCTCTATTGATGTTCATGAATGTCATCGTACTCTTAAGGTTTC

-2334 TGAATATGCGATTAAGTACACTGGAAGAATTACTGATATTGTTGGCAATGTGC**AAATTAC**

WUN

-2274 CTA**TGAC**GTGA**C**CATAAAGGGCACGATCAATGAGCTTAGGTAGCTCAGGCTGAAATAGCC

Asl/TGA

-2214 A**AGAAAAGGT**AAAAGGTTTGCAGTGGCAT**CAACTG**ATTGATAAGCTTGCAGCGGCAACTT

HSE **MBS**

-2154 GGTTCTTTTAAGTGATTTTGATTGATCGTTGTGTTGATC**TTATTTCAAA**AGAGTGATTAT

WUN/ERE

-2094 GAGTTTTTATTTGTGTTTTTTTTTAT**CGGTCA**TTTGTTGTGGCAGATTTTATGAAAGATTA

MBS

-2034 TAC**TTGGAAATGCTT**AGTTAATAACGTATCCTCAATTCCTCAACCTGGTAGCCGTTTCTT

INVERTED repeat (a)

-1974 **GAC**GTTTTGATTGTTTCTATTATTATTCGGTATTTTCTTAAGTCACTCACTGAGCATTAG

TGA element

-1914 TTTGCTCAT**CCCTTTTT**GTGTTTTCTGTTTGCATGTCTGTTAGTTT**CCTTTT**CAGAAGTG

P-box **P-box**

-1854 TTCGGGTTGTGTGTAGCTTAGAG[redacted]TGGTTCACTTGTTTAAGAGAAAG

15bp DYAD (a) | DIRECT REPEAT (a)

INVERTED REPEAT (b)

-1794 TGGTTCACTTGTTTAAAAGAAAGGGCTTTCTCTTATAAAACTGATTTGGATTTTATTTGT

| DIRECT REPEAT (a)

-1734 **AAATTACT**TCTGATGTGCTCATTTTCTCTACCTTTATATTTATAGAGAATTTACTTGCTT

WUN

-1674 ATATCTTTTTGTATTTA**GAATTTCAT**TTTTGAGATTTAATAATGCTATTGTCCATTTTGG

WUN

-1614 GACTGGGTCGTTTCATGCTTTATAGTCCAAAGCAGTCAAGATGGGTGTATTTATTAC**CCG**

LTR

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Fig. 1a

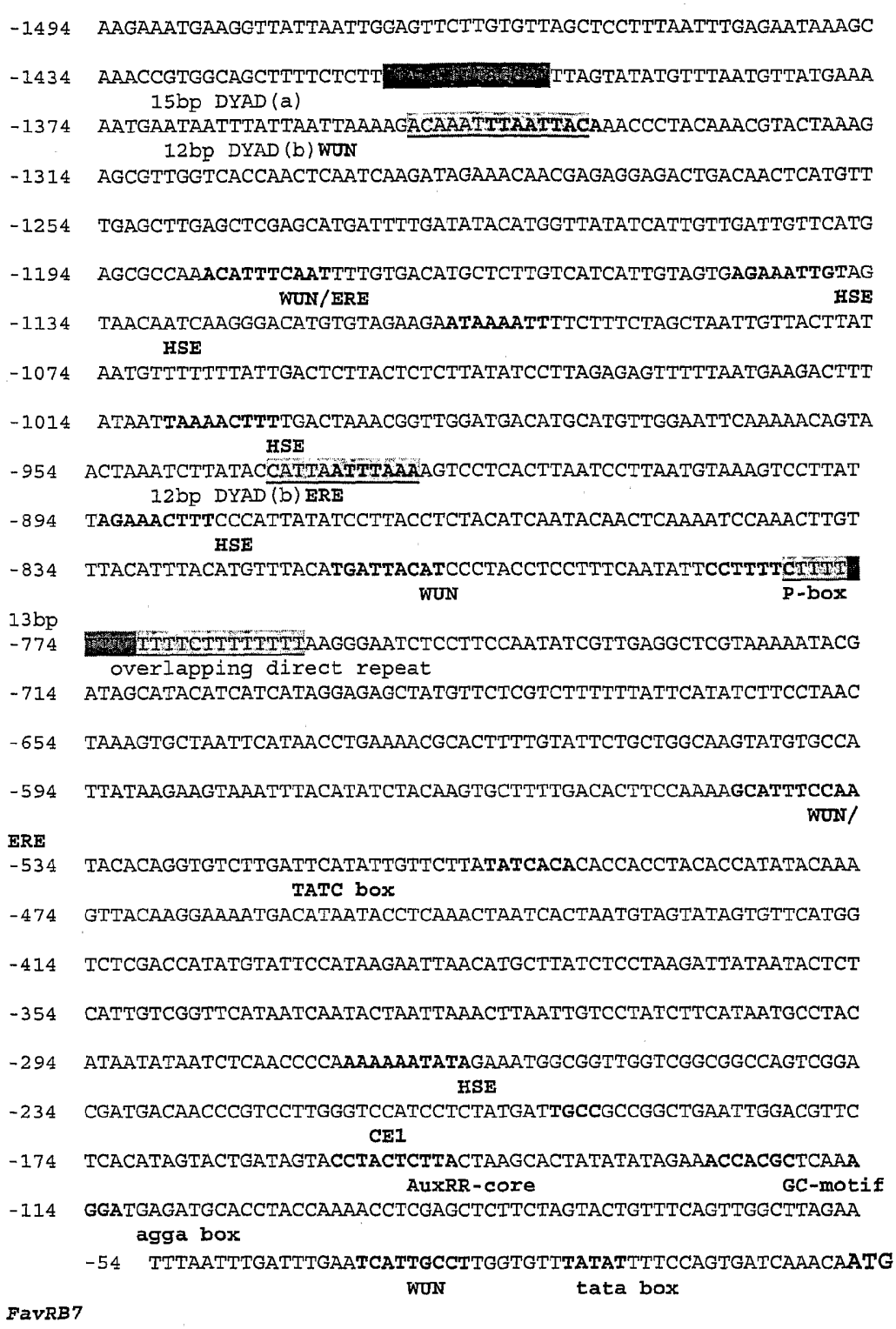

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15bp DYAD(a)
-1374 AATGAATAATTTATTAATTAAAAGACAAATTTAATTACAAACCCTACAAACGTACTAAAG
12bp DYAD(b) WUN
-1314 AGCGTTGGTCACCAACTCAATCAAGATAGAAACAACGAGAGGAGACTGACAACTCATGTT
-1254 TGAGCTTGAGCTCGAGCATGATTTTGATATACATGGTTATATCATTGTTGATTGTTCATG
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WUN/ERE HSE
-1134 TAACAATCAAGGGACATGTGTAGAAGAATAAAATTTTCTTTCTAGCTAATTGTTACTTAT
HSE
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HSE
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12bp DYAD(b) ERE
-894 TAGAAACTTTCCCATTATATCCTTACCTCTACATCAATACAACTCAAAATCCAAACTTGT
HSE
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WUN P-box
13bp
-774 TTTTTTTTTTTTTTAAGGGAATCTCCTTCCAATATCGTTGAGGCTCGTAAAAATACG
overlapping direct repeat
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-594 TTATAAGAAGTAAATTTACATATCTACAAGTGCTTTTGACACTTCCAAAAGCATTTCCAA
WUN/
ERE
-534 TACACAGGTGTCTTGATTCATATTGTTCTTATATCACACACCACCTACACCATATACAAA
TATC box
-474 GTTACAAGGAAAATGACATAATACCTCAAACTAATCACTAATGTAGTATAGTGTTCATGG
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HSE
-234 CGATGACAACCCGTCCTTGGGTCCATCCTCTATGATTGCCGCCGGCTGAATTGGACGTTC
CE1
-174 TCACATAGTACTGATAGTACCTACTCTTACTAAGCACTATATATAGAAACCACGCTCAAA
AuxRR-core GC-motif
-114 GGATGAGATGCACCTACCAAAACCTCGAGCTCTTCTAGTACTGTTTCAGTTGGCTTAGAA
agga box
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WUN tata box
FavRB7

Fig. 1b

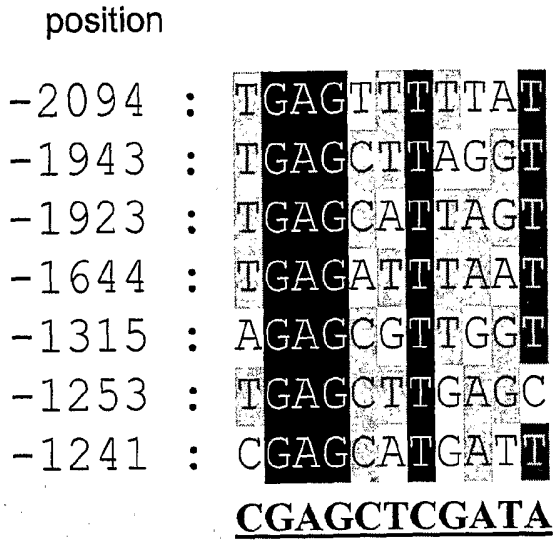

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Fig. 2

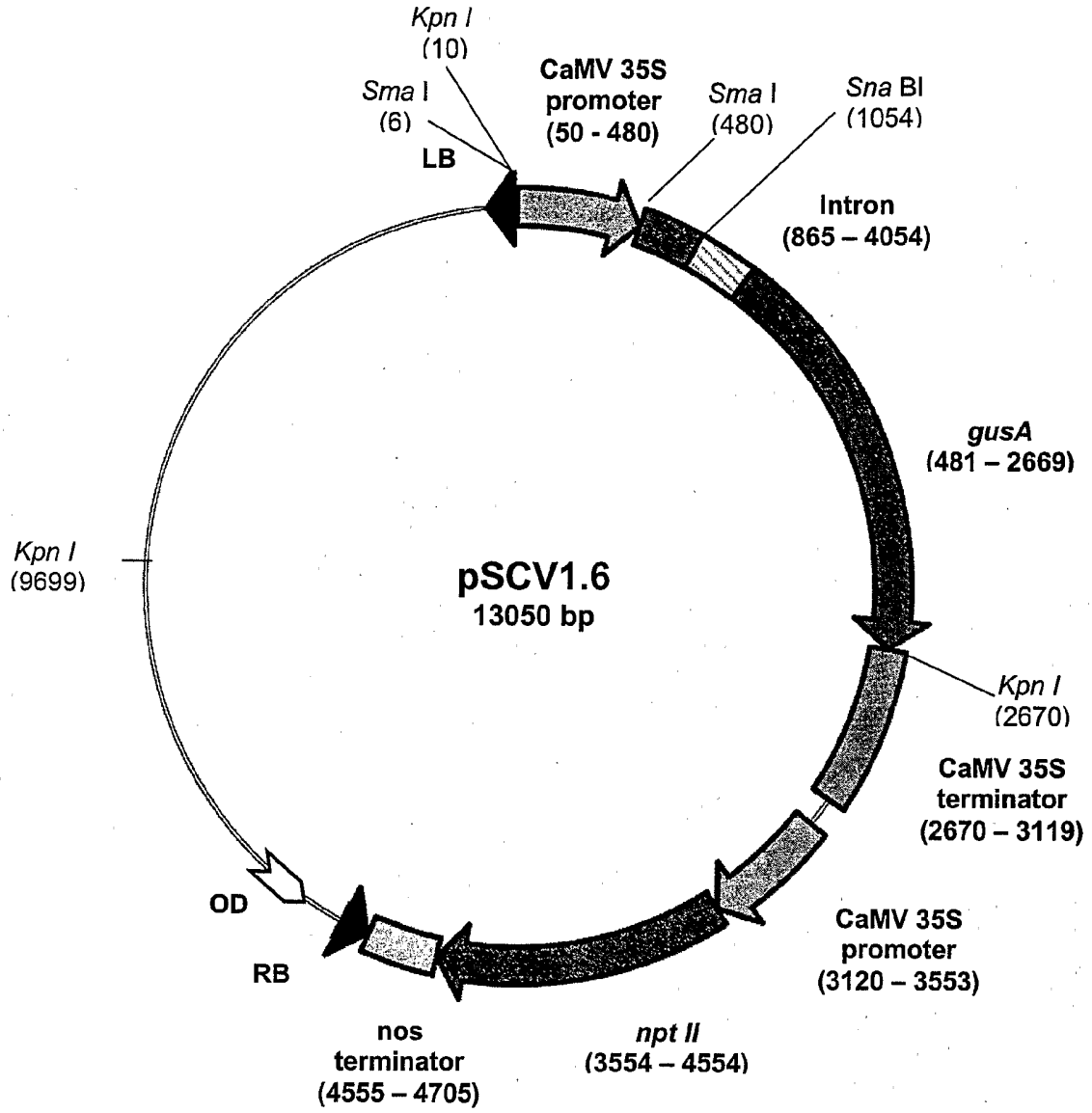

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(10)
Sma I
(6)
LB
CaMV 35S
promoter
(50 - 480)
Sma I
(480)
Sna BI
(1054)
Intron
(865 – 4054)
gusA
(481 – 2669)
Kpn I
(2670)
CaMV 35S
terminator
(2670 – 3119)
CaMV 35S
promoter
(3120 – 3553)
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Fig. 3

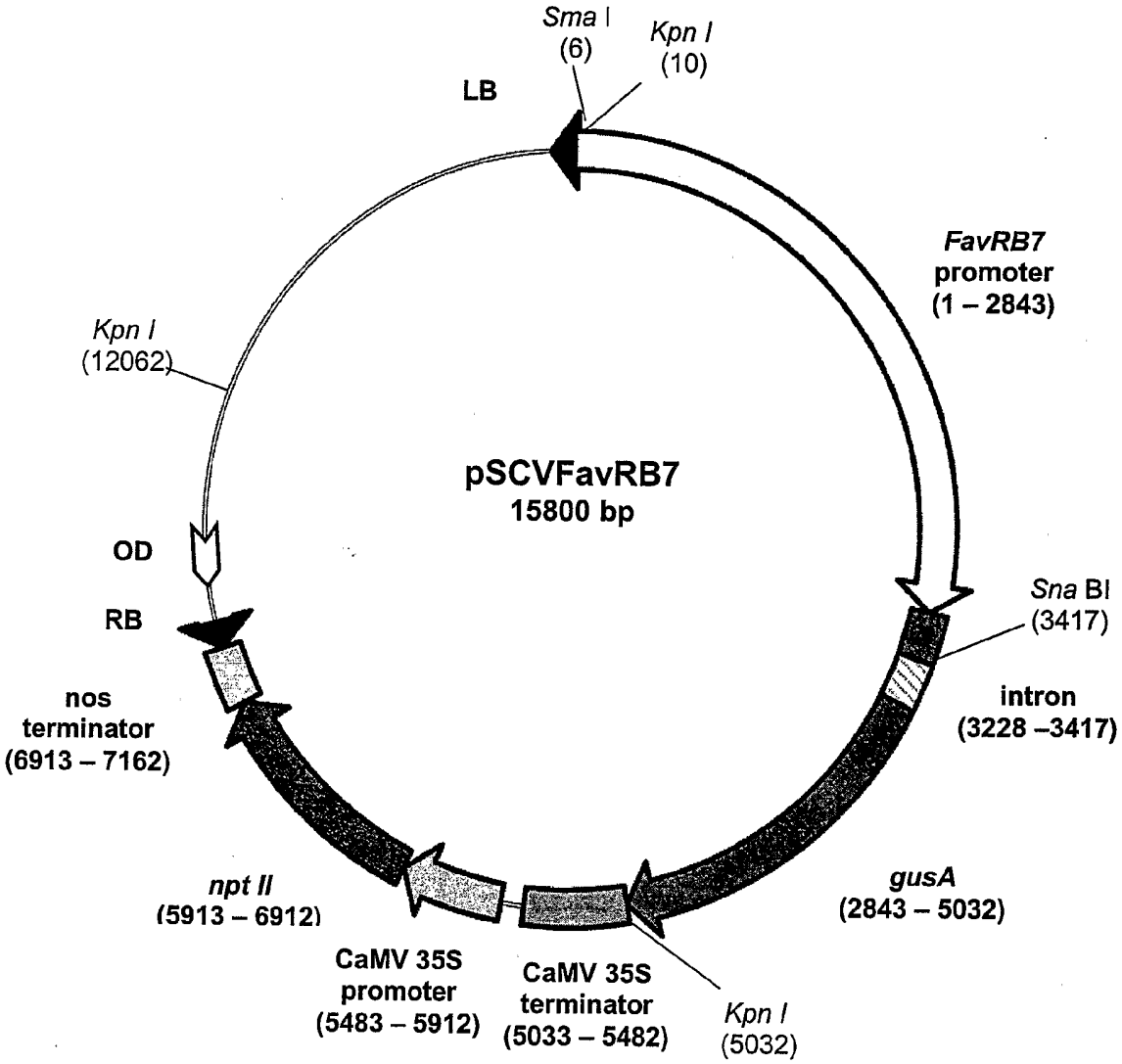

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Kpn I
(10)
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promoter
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(12062)
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OD
RB
Sna BI
(3417)
intron
(3228 –3417)
nos
terminator
(6913 – 7162)
gusA
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npt II
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Fig. 4

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<151> 2003-05-30

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/GB2004/002334

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 459 252 A (CONKLING MARK A ET AL) 17 October 1995 (1995-10-17) abstract; examples 2,3 column 2, line 50 - column 3, line 10	1-36
Y	YAMAMOTO Y T ET AL: "CHARACTERIZATION OF CIS-ACTING SEQUENCES REGULATING ROOT-SPECIFIC GENE EXPRESSION IN TOBACCO" PLANT CELL, vol. 3, no. 4, 1991, pages 371-382, XP002295005 ISSN: 1040-4651 abstract; figures 1,8	1-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2004	01/10/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Loubradou, G

INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
PCT/GB2004/002334

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SARDA XAVIER ET AL: "Characterization of closely related delta-TIP genes encoding aquaporins which are differentially expressed in sunflower roots upon water deprivation through exposure to air" PLANT MOLECULAR BIOLOGY, vol. 40, no. 1, 1 May 1999 (1999-05-01), pages 179-191, XP002295006 ISSN: 0167-4412 abstract; figures 2,3,5	1-36
A	OPPERMAN C H ET AL: "ROOT-KNOT NEMATODE-DIRECTED EXPRESSION OF A PLANT ROOT-SPECIFIC GENE" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 263, 14 January 1994 (1994-01-14), pages 221-223, XP002922695 ISSN: 0036-8075 the whole document	1-36
A	DATABASE EMBL 29 January 1997 (1997-01-29), KAWAHARA: "Carrot mRNA for root specific gene, complete cds" XP002295256 Database accession no. AB000506 the whole document	1-36
A	WO 99/43797 A (PIONEER HI BRED INT ; RICE DOUGLAS A (US)) 2 September 1999 (1999-09-02) abstract; sequences 1-10	
A	WO 97/28268 A (FOSTER ATKINSON ELIZABETH ; JAMES ELIZABETH EVA (CA); FOBERT PIERRE (C) 7 August 1997 (1997-08-07) abstract; sequence 1	
A	ATKINSON HOWARD J ET AL: "Engineering plants for nematode resistance." ANNUAL REVIEW OF PHYTOPATHOLOGY. 2003, vol. 41, 1 May 2003 (2003-05-01), pages 615-639, XP002295007 ISSN: 0066-4286 the whole document	
A	WO 01/98480 A (SYNGENTA PARTICIPATIONS AG ; BROWN DEVON (US); BUDWORTH PAUL (US); COO) 27 December 2001 (2001-12-27) the whole document	
A	WO 03/040322 A (SYNGENTA PARTICIPATIONS AG ; SKALLA DALE (US); LAWTON KAY (US); MILLIG) 15 May 2003 (2003-05-15) the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ›nal Application No
PCT/GB2004/002334

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5459252	A	17-10-1995	NONE		
WO 9943797	A	02-09-1999	AU	762993 B2	10-07-2003
			AU	2880399 A	15-09-1999
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			ZA	9700837 A	04-08-1997
			US	2004073022 A1	15-04-2004
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			EP	1294914 A2	26-03-2003
			WO	0198480 A2	27-12-2001
WO 03040322	A	15-05-2003	CA	2462615 A1	15-05-2003
			WO	03040322 A2	15-05-2003

INTERNATIONAL SEARCH REPORT

ıational application No.

PCT/GB2004/002334

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] In written format

   [X] In computer readable form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in computer readable form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: