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(54) **PRODUCTION DE BRASSICA HYBRIDES AUTO-
COMPATIBLES AU MOYEN D'UN SYSTEME DE
REGULATION DE LA POLLINISATION AUTO-
INCOMPATIBLE**

(54) **PRODUCTION OF SELF-COMPATIBLE BRASSICA HYBRIDS
USING A SELF-INCOMPATIBLE POLLINATION CONTROL
SYSTEM**

(57) L'invention concerne des cellules d'une plante hybride auto-compatible, dont les parents sont un parent femelle homozygote auto-incompatible et un parent mâle homozygote et dont le génome nucléaire contient un vecteur comprenant une séquence d'ADN qui, lorsqu'elle est exprimée dans une cellule, confère un auto-incompatibilité à la plante, et un promoteur capable de diriger l'expression de la séquence d'ADN dans la cellule. L'invention porte également sur le vecteur, sur une plante comprenant les cellules, sur une plante transformée à l'aide dudit vecteur, sur les graines desdites plantes et sur un procédé permettant de conférer le phénotype auto-compatible à la progéniture du parent femelle auto-incompatible et du parent mâle auto-compatible. Elle se rapporte enfin à un système de régulation d'auto-pollinisation pour la production ou la sélection de graines hybrides, qui, grâce à un transgène de suppression permettant d'éliminer le phénotype d'auto-incompatibilité dans des hybrides, peut augmenter le rendement.

(57) This invention relates to cells of a self-compatible hybrid plant, the parents of which are a homozygous self-incompatible female parent, and a homozygous male parent, and the nuclear genome of which contains a vector comprising a DNA sequence which, when expressed in a plant cell, imparts self-incompatibility to the plant, and a promoter capable of directing the expression of the DNA sequence in the cell. The invention includes the vector, a plant comprising the cells, a plant transformed with the vector, the seed of such plants, and a method for conferring the self-compatible phenotype on progeny of the self-incompatible female parent and self-compatible male parent. The invention comprises an improved self-pollination control system for hybrid seed production or breeding which, by using a knockout transgene to eliminate the self-incompatibility phenotype in hybrids, can result in increased yields.

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(21) International Application Number: PCT/CA98/00089 (22) International Filing Date: 4 February 1998 (04.02.98) (30) Priority Data: 60/037,719 7 February 1997 (07.02.97) US (71) Applicant (for all designated States except US): PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; Darwin Building, 7100 N.W. 62nd Avenue, P.O. Box 1000, Johnston, IN 50131 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ROTHSTEIN, Steven, J. [CA/CA]; 15 Fieldstone Road, Guelph, Ontario N1L 1A5 (CA). STAHL, Richard, J. [CA/CA]; 66 Sheridan Street, Guelph, Ontario N1E 3T8 (CA). GORING, Daphne, R. [CA/CA]; 12 Webster Drive, Aurora, Ontario L4G 2A5 (CA). (74) Agent: DEETH WILLIAMS WALL; Suite 400, 150 York Street, Toronto, Ontario M5H 3S5 (CA).		(81) Designated States: AU, CA, CZ, JP, MX, NO, NZ, PL, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: PRODUCTION OF SELF-COMPATIBLE <i>BRASSICA</i> HYBRIDS USING A SELF-INCOMPATIBLE POLLINATION CONTROL SYSTEM (57) Abstract This invention relates to cells of a self-compatible hybrid plant, the parents of which are a homozygous self-incompatible female parent, and a homozygous male parent, and the nuclear genome of which contains a vector comprising a DNA sequence which, when expressed in a plant cell, imparts self-incompatibility to the plant, and a promoter capable of directing the expression of the DNA sequence in the cell. The invention includes the vector, a plant comprising the cells, a plant transformed with the vector, the seed of such plants, and a method for conferring the self-compatible phenotype on progeny of the self-incompatible female parent and self-compatible male parent. The invention comprises an improved self-pollination control system for hybrid seed production or breeding which, by using a knockout transgene to eliminate the self-incompatibility phenotype in hybrids, can result in increased yields.		

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PRODUCTION OF SELF-COMPATIBLE BRASSICA HYBRIDS
USING A SELF-INCOMPATIBLE POLLINATION CONTROL SYSTEM

Field of invention

5 This invention relates to cells of a self-compatible hybrid plant, the parents of which are a homozygous self-incompatible female parent and a homozygous male parent. The nuclear genome of the male parent contains a vector comprising a DNA sequence which, when expressed in a plant cell, imparts self-incompatibility to the plant and a promoter capable of directing the expression of the DNA sequence in
10 the cell. The invention includes the vector, a plant comprising the cells, a plant transformed with the vector, the seed of such plants, and a method for conferring the self-compatible phenotype on progeny of the self-incompatible female parent and self-compatible male parent. The invention comprises an improved self-incompatibility pollination control system for hybrid seed production or breeding
15 which, by using a knockout transgene to eliminate the self-incompatibility phenotype in hybrids, can result in increased yields.

Background of the Invention

Canola

Seed from *Brassica* plants is an increasingly important crop. As a source of
20 vegetable oil, it presently ranks behind only soybeans and palm in commercial importance and it is comparable with sunflowers. The oil is used both as a salad oil and as a cooking oil.

In its original form, *Brassica* oil, known as rapeseed oil, was harmful to humans due to its relatively high level of erucic acid. Erucic acid is commonly present in native
25 cultivars in concentrations of 30 to 50 percent by weight based upon the total fatty acid content. This problem was overcome when plant scientists identified a germplasm source of low erucic acid rapeseed oil (Stefansson, 1983).

In addition, plant scientists have attempted to improve the fatty acid profile for rapeseed oil (Robbelen, 1984; Ratledge et al., 1984; Robbelen et al., 1975; and
30 Rakow et al., 1973). These references are representative of those attempts.

Particularly attractive to plant scientists were so-called "double-low" varieties: those low in erucic acid in the oil and low in glucosinolates in the solid meal remaining

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after oil extraction (i.e., an erucic acid content of less than 2 percent by weight based upon the total fatty acid content, and a glucosinolate content of less than 30 μ mol/gram of the oil-free meal). These higher quality forms of rape, first developed through plant breeding in Canada, are known as canola.

5 **Plant Breeding**

The goal of plant breeding is to combine in a single variety or hybrid various desirable traits of the parental lines. For field crops, these traits may include resistance to diseases and insects, tolerance to heat and drought, reducing the time to crop maturity, greater yield, and better agronomic quality. With mechanical harvesting
10 of many crops, uniformity of plant characteristics such as germination and stand establishment, growth rate, maturity, and size, is important.

Field crops are bred through techniques that take advantage of the plant's method of pollination. A plant is self-pollinating if pollen from one flower is transferred to the same or another flower of the same plant. A plant is cross-pollinating if the
15 pollen comes from a flower on a different plant.

Plants that have been self-pollinated and selected for many generations become homozygous at almost all gene loci and produce a uniform population of true breeding progeny. A cross between two homozygous lines produces a uniform population of hybrid plants that may be heterozygous for many gene loci. A cross of
20 two plants each heterozygous at a number of gene loci will produce a population of hybrid plants that differ genetically and will not be uniform.

The development of hybrids requires the development of homozygous inbred lines, the crossing of these lines and the evaluation of the crosses. Pedigree breeding and recurrent selection are two of the breeding methods used to develop inbred lines
25 from populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which new inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential.

30 Pedigree breeding starts with the crossing of two genotypes, each of which may have one or more desirable characteristics that is lacking in the other or which complement the other. If the two original parents do not provide all of the desired

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characteristics, other sources can be included in the breeding population. In the pedigree method, superior plants are selfed and selected in successive generations. In the succeeding generations the heterozygous condition gives way to homogeneous lines as a result of self-pollination and selection. Typically in the pedigree method of breeding five or more generations of selfing and selection is practiced.

A hybrid variety is the cross of two inbred lines, each of which may have one or more desirable characteristics lacked by the other or which complement the other. The hybrid progeny of the first generation is designated F_1 . In the development of hybrids, only the F_1 hybrid plants are sought. The F_1 hybrid is more vigorous than its inbred parents. This hybrid vigor, or heterosis, can be manifested in many ways, including increased vegetative growth and increased yield.

The development of a hybrid variety involves three steps: (1) the selection of superior plants from various germplasm pools; (2) the selfing of the superior plants for several generations to produce a series of inbred lines, which although different from each other, each breed true and are highly uniform; and (3) crossing the selected inbred lines with unrelated inbred lines to produce the hybrid progeny (F_1). During the inbreeding process the vigor of the lines decreases. Vigor is restored when two unrelated inbred lines are crossed to produce the hybrid progeny (F_1). An important consequence of the homozygosity and homogeneity of the inbred lines is that the hybrid between any two inbreds will always be the same. Once the inbreds that give the best hybrid have been identified, the hybrid seed can be reproduced indefinitely as long as the homogeneity of the inbred parents is maintained.

A single cross hybrid is produced when two inbred lines are crossed to produce the F_1 progeny. A double cross hybrid, is produced from four inbred lines crossed in pairs ($A \times B$ and $C \times D$) and then the two F_1 hybrids are crossed again $(A \times B) \times (C \times D)$. Much of the hybrid vigor exhibited by F_1 hybrids is lost in the next generation (F_2). Consequently, seed from hybrid varieties is not used for planting stock. Likewise, it is very important in the production of hybrid seed to avoid self-pollination of the inbreds and the production and sale of inbred seed to end users.

Hybrid production among self-pollinated crops can be difficult because of the close association of the male and female reproductive organs. In addition to the physical difficulty in effecting hybrid production in a self-pollinating crop, the amount of

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heterosis exhibited in a hybrid is often too low to justify the additional expense required to produce hybrid seed. A reliable form of male sterility offers the opportunity for improved hybrid plant breeding and increased yields.

Male Sterility and Hybrids

5 In developing improved new *Brassica* varieties, breeders use self-incompatible (SI), cytoplasmic male sterile (CMS) and nuclear male sterile (NMS) *Brassica* plants as the female parent. In using these plants, breeders are attempting to improve the efficiency of seed production and the quality of the F₁ hybrids and to reduce the breeding costs. When hybridisation is conducted without using SI, CMS or NMS
10 plants, it is more difficult to obtain and isolate the desired traits in the progeny (F₁ generation) because the parents are capable of undergoing both cross-pollination and self-pollination. If one of the parents is an SI, CMS or NMS plant that is incapable of producing pollen, only cross pollination will occur. By eliminating the pollen of one parental variety in a cross, a plant breeder is assured of obtaining hybrid seed of
15 uniform quality, provided that the parents are of uniform quality and the breeder conducts a single cross.

SI and Hybrids

As indicated above, there are a variety of mechanisms that promote out-crossing in plants, one of these being self-incompatibility (SI). In self-incompatible
20 species, self-pollen is rejected, thus ensuring that most fertilization involves pollen produced by a different plant. In *Brassica* species, self-incompatibility is of the sporophytic type in which the pollen parent determines the phenotype of the pollen.

In SI pollination control systems in *Brassica*, the stigma papillae cells must be able to differentiate between self-pollen and pollen derived from parents carrying
25 different alleles associated with the expression of the SI phenotype. Once this recognition event occurs, it sets in motion a train of physiological events that prevents the germination of self- pollen, while allowing the germination and subsequent fertilization by pollen from a plant carrying different alleles associated with the expression of the SI phenotype. This event happens when both types of pollen are
30 present on the stigma surface (see WO94/09139, for example).

SI Alleles

There are a large number of self-incompatible alleles that have been identified

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genetically. In SI plants, pollen derived from a plant that carries the same allele as that present in the stigma are rejected (deNattancourt, 1977). These alleles are generally co-dominant with each other and self-incompatibility is dominant over self-compatibility (Ockenden, 1974).

5 SI in *Brassica* is controlled by a single dominant genetic locus called the S-locus (Bateman, 1955). The sporophytic nature of this incompatibility system results in the pollen phenotype being derived from the genotype of the diploid pollen parent and not from the haploid pollen genotype. Several genes from the S-locus have been isolated and characterized (see WO94/09139, for example). Recent efforts have
10 focused on transforming self-compatible *Brassica* lines with these genes to produce a self-incompatibility phenotype.

Brassica napus is an amphidiploid plant which is normally self-compatible while its presumptive progenitors, *Brassica oleracea* and *Brassica rapa*, are diploid self-incompatible species. Self-incompatible alleles can be transferred into *Brassica napus*
15 from the progenitor species via inter-specific crosses (Goring et al, 1992a; 1992b) creating self-incompatible lines. These are then repeatedly back-crossed to the *Brassica napus* parent (Goring et al, 1992a; 1992b).

The recognition of self-pollen and its subsequent rejection is a very rapid process. For strong S-alleles, like the 910 allele, very little of the pollen will even
20 hydrate thus preventing subsequent germination. The pollen that does germinate forms tubes which grow for a short distance and never penetrate the stigma surface. Given the sporophytic nature of *Brassica* SI, it has been postulated that some factor is produced in the tapetum or in the microspores of the parental plant, that this factor is present on the outer surface of the pollen exine and is recognized by something on
25 the stigma surface which prevents the normal pollen germination and fertilization process from occurring (Goring and Rothstein, 1992).

There have been two genes that have been found to be associated with the self-incompatible phenotype. The first to be discovered codes for the S-locus glycoprotein (SLG) which is expressed primarily in stigma papillae cells although
30 some transcript is detected in anther tissue (Sato et al, 1991). The sequence of the SLG genes from different S-loci vary considerably (80-90% similarity), which is reasonable for a protein that might be involved in self-recognition. The second gene

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codes for a protein called the S-Receptor Kinase (SRK) (Stein et al, 1991). This is a protein with a receptor-like domain very similar in sequence to the SLG, a trans-membrane domain and a kinase domain that has been shown to have serine-threonine kinase activity (Goring and Rothstein, 1992). The receptor portion has some
5 similarity in sequence to immunoglobulin-like receptor domains in animal cells (Glavin et al, 1994) and shows a similar sequence heterogeneity between genes derived from different alleles as that seen for the SLG. This gene is also expressed in the stigma papillae cells (Stein et al, 1991; Goring and Rothstein, 1992).

Ways in Which SI Works

10 Based on experimental observations and on what is known about receptor-kinase ligand interactions in mammalian cells, it is possible that the SRK protein forms a receptor complex in the stigma papillae cells which recognizes an allele specific ligand present on the pollen surface. The SLG is either involved in bringing the ligand into contact with the SRK or serve as part of the receptor complex. The pollen ligand
15 might be a protein coded for by a separate linked gene and be recognized only by the receptor complex coded for by the same allele. Alternatively, the ligand could be the SLG itself activated in the pollen by the binding of another molecule.

Involvement of the SLG and SRK in the SI response has been supported by their linkage to the S-locus and also on the analysis of plants selected for self-
20 compatibility, with these turning out to not express either the SLG (Nasrallah, 1992) or SRK (Nasrallah et al, 1994) at normal levels. In addition, self-compatible *Brassica napus* has an S-allele with a SRK gene having a 1 bp deletion which would code for a truncated protein product (Goring et al, 1993). However, in each of these cases, it cannot be definitively demonstrated that changes in other genes were not involved in
25 the change in phenotype. Finally, when a wild-type SLG gene from *B. campestris* was transformed into a self-incompatible *B. oleracea* line, this was reported to disrupt the self-incompatible phenotype (Toriyama et al, 1992).

SI Pollination Control Systems

A variety of traditional techniques have been used to attempt to overcome self-
30 incompatibility of hybrids produced using SI pollination control systems. These are micropropagation, variation of levels of carbon dioxide and other chemical treatments and bud pollination (see, for example, US Patent 3,043,282 (Pearse); US Patent

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4,499,687, (Lawrence et al.)). However, these traditional techniques cannot be used on a field scale. Extensive field production of the hybrids using the SI line as female parent is impractical due to the production costs.

For example, Canadian patent application 2,143,781 of Yamashita, et al., published on September 11, 1995, claims a hybrid breeding method for crop plants in the family *Brassicaceae* in which an F_1 seed is produced by crossing the female parent of a self-incompatible male sterile line with a male parent. In one embodiment, the male parent possesses a fertility restoring gene. The fertility restoring gene (IM-B) is for MS-N1-derived cytoplasm with male sterility (CMS) and was derived from a winter variety (IM line). This was then crossed with a spring double-low line (62We). This restorer is derived from traditional breeding techniques rather than from a construct containing a transgene.

Recent research efforts have been made to isolate genes for SI and to introduce them, via known procedures, into elite seed rape lines. In these efforts, persons have isolated cDNA associated with the self-incompatibility phenotype in plants and to confer the phenotype on self-compatible plants. Efforts have not focussed on using molecular methods to confer a self-compatible phenotype on progeny of a self-incompatible plant.

For example, WO 9409139 of Pioneer Hi-Bred International, Inc. and the University of Guelph claims an isolated cDNA encoding for the S-locus receptor kinase-910 protein or a kinase active fragment. The sequence is useful for screening the progeny for the SI phenotype or to confer the SI phenotype on a self-compatible plant. The invention is directed towards determining the presence of the self-incompatibility phenotype rather than eliminating the self-incompatibility phenotype in hybrids.

WO 9318149 of Pioneer Hi-Bred International, Inc. and the University of Guelph claims isolated cDNAs of the self-incompatibility loci of *Brassica napus* ssp. *rapifera* and *Brassica campestris*; methods for identifying, amplifying or detecting these sequences or for conferring the SI phenotype on a self-compatible plant. Again, the invention is directed towards determining the presence of the self-incompatibility phenotype rather than eliminating the self-incompatibility phenotype in hybrids.

Canadian patent application 2,071,473 of Ciba-Geigy AG relates to an isolated

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DNA molecule encoding an SRK polypeptide having an S-locus binding domain, a transmembrane domain and a protein kinase domain, a method of isolating and identifying an SRK gene. Again, this invention does not attempt to improve SI pollination control systems.

5 Toriyama et al., 1992 reported that the introduction of an SLG gene into a self-incompatible hybrid perturbed the self-incompatibility phenotype of stigma and pollen and resulted in the production of a self-compatible phenotype. While this construct involved the use of a transgene to knock out incompatibility, (1) it did not extend to use of transgenes other than SLG (such as SRK), (2) it did not involve use of a vector
10 comprising a mutant or antisense DNA sequence, and (3) it was not directed to eliminating the self-incompatibility phenotype in hybrids for breeding purposes. It simply involve the transformation by *Agrobacterium* of a wild-type SLG gene from a *B. campestris* plant into a self-incompatible *B. oleracea* plant, which was left to pollinate over night and the objective of the work was simply to find some evidence that pollen-
15 stigma interaction of incompatibility is influenced by the expression of the SLG gene.

Need for Improved SI Pollination Control System

 In SI Pollination control systems, production of F₁ hybrids includes crossing an SI *Brassica* female parent, with a pollen producing male *Brassica* parent. As indicated above, in an SI pollination control system, the pollen phenotype is derived
20 from the genotype of the diploid pollen parent and not from the haploid pollen genotype. Thus, a hybrid produced from a homozygous SI female parent and a homozygous self-compatible male parent is heterozygous for SI.

 There are a number of difficulties in relying upon a hybrid line that is heterozygous for SI. These difficulties are that: (1) heterozygous SI alleles do not
25 provide sufficient pollination control, (2) *Brassica napus* is a self-pollinating species, so under poor pollination conditions (such as prolonged cool, wet weather) there may be inadequate pollen movement from the male fertile plants to the F₁ hybrid plants, resulting in poor seed set and yield, and (3) the F₁ hybrid plants are more vigorous than inbred plants, which may be used to pollinate self-incompatible hybrids, so the
30 former may outcompete the latter, resulting in too little pollen being available for optimal seed set and yield on the F₁ plants. This leads to a considerable decrease in yield.

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Efforts to date have focused on either (1) attempting to overcome self-incompatibility of hybrids using traditional techniques or (2) attempting to confer and strengthen self-incompatibility phenotypic expression using molecular methods. No attempts have been made to address the need of conferring a self-compatibility phenotype in hybrid progeny of self-incompatible plants. Thus, there is a need to develop an SI pollination control system which addresses this need.

To date, no breeder has commercialized an SI pollination control system in *Brassica* in which the female parent is homozygous self-incompatible, the male parent is homozygous self-compatible and the hybrid has a self-compatible phenotype. Breeders could use the improved SI pollination control system to produce *Brassica* hybrids which have self-compatible phenotypes and are self pollinating. This would benefit farmers, who could then plant *Brassica* hybrids which, following self-pollination, would yield seed having desirable characteristics. This would also simplify breeding programs and, by allowing self-pollination, would increase yields. Such an improved SI pollination control system would also be useful in producing self-compatible hybrids of species other than *Brassica* which use an SRK transgene to knockout self-incompatibility.

It is an object of the present invention to provide a method for producing self-compatible hybrids by cross-breeding homozygous self-compatible male parent plants with homozygous self-incompatible female parent plants into which has been transformed a vector comprising a promoter and a DNA sequence which imparts a phenotype for self-compatibility. This invention relates to such a method in species of plants which use an SRK gene to knock out their self-incompatibility, including plants of the genera *Brassica*. It also discloses cells and seeds of the self-compatible hybrid plants produced, the plants comprising the cells, the vectors transformed into the self-incompatible parent plant and the plants transformed with such vectors.

These and other objects and advantages of the invention will be apparent to those skilled in the art from a reading of the following description and appended claims.

Summary of the Invention

Brief Description of the Drawings

The invention will now be described in relation to the figures in which:

FIG. 1 illustrates the regulatory regions used to construct A10-mutant SRK and SLR1-mutant SRK as described in Example 1

FIG. 2 illustrates an analysis of the expression of the 910 SRK, 910-SLG and A10 SLG transcripts in the A10-mutant SRK self-compatible line.

5 FIG. 3 illustrates the expression of the A14 allele that is present in the T2 line and is also decreased.

FIG. 4 illustrates an analysis of the 910 SRK , 910 and A10 SLG transcripts -in the SLR1-mutant SRK self-compatible line.

10 FIG. 5 illustrates the A10-antisense SRK, the SLR1-antisense SRK and the TA39-antisense chimeric genes as described in Example 7.

15 FIG. 6A-C present the nucleotide sequence and predicted amino acid sequence of the mutant SRK-910 gene. The underlined sections represent the signal peptide and transmembrane domain. Conserved cysteine residues are marked by a dash above the amino acid residue. Potential N-glycosylation sites are represented by bold-italic type. FIG. 6C shows the mutant SRK-910 gene with additional untranslated nucleotides.

Description of Preferred Embodiments

20 In mammalian cells considerable work has been done on tyrosine receptor kinases (Ulrich and Schesinger, 1990). In these cases, the binding of the specific ligand leads to the dimerization of the receptor kinase and thus stimulates kinase activity which in turn leads to the change in phenotype through a variety of signal transduction processes. In a number of cases, the expression of a mutant receptor kinase turned out to be a dominant-negative mutation due to the formation of inactive dimers between the mutant and wild-type proteins. Previously, we had shown that a
25 mutation in the kinase domain of the SRK prevented kinase activity when the kinase domain of the SRK was expressed in bacteria (Goring and Rothstein, 1992) and we therefore made a single base-pair mutation at this site in the 910-SRK and transformed this mutant gene into the W1 line which carries this allele. Two SC transgenic lines were found in which only the stigma side of the SI phenotype was
30 affected and in which this phenotype segregated genetically with the transgene. This work strongly supports the importance of the SRK for the stigma side of the SI phenotype.

The following Examples are presented as specific illustrations of the present invention. It should be understood, however, that the invention is not limited to the specific details set forth in the Examples.

Example 1: Self-Incompatible Lines

5 *Brassica napus* is an amphidiploid plant which is normally self-compatible while its presumptive progenitors, *Brassica oleracea* and *Brassica rapa*, are diploid self-incompatible species. Self-incompatible alleles can be transferred into *Brassica napus* from the progenitor species via inter-specific crosses (Goring et al 1992a; 1992b) creating self-incompatible lines. These were then repeatedly back-crossed to the
10 *Brassica napus* parent (Goring et al, 1992a; 1992b). The two self-incompatible lines used in this study are called (1) W1 which carries the 910 allele, and (2) T2 which carries the A14 allele. Other than the region involved in self-incompatibility, these lines are virtually isogenic pairs with the original self-compatible lines used as the recurrent parent (for these lines W1 is similar to Westar and T2 is similar to Topas).

15 **Example 2: The chimeric mutant SRK genes and plant transformation**

The kinase coding region of the 910 SRK gene was modified so that the codon at position 557 coded for an alanine instead of a lysine. This substitution had earlier been shown to prevent kinase activity completely when it was expressed in *E. coli* (Goring and Rothstein, 1992). The mutated 910-SRK was then reconstructed so that
20 the only difference between the wild-type and mutant versions was this one base pair substitution. Our assumption was that this mutant version would be able to be assembled into a receptor complex, but that the deficiency in kinase activity would prevent it from functioning.

Two regulatory regions were used to express the mutant 910-SRK as shown in
25 Figure 1. The first utilized the promoter from the SLG A10 gene which is expressed at very high levels in stigma tissue, which we called the "A10-mutant SRK" (Goring et al, 1992b). The promoter for this gene was isolated using standard molecular techniques (Sambrook et al, 1989) and includes 1.7 kb of the region upstream of the transcription initiation site. The second was the promoter from the SLR1 gene which is also
30 expressed at high levels in stigma tissue, which we called the "SLR1-mutant SRK" (Franklin et al, 1996) and had been shown to work well in transgenic plants. We were attempting to determine whether expressing the mutant 910-SRK at high levels would

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have a greater chance of altering phenotype.

These chimeric genes were transformed into the self-incompatible cultivar W1 (similar to Westar and which carries the 910 allele) and the resulting transformants were analyzed for their ability to set self seed. Of the 50 transformants that were transformed with the A10-mutant SRK construct, only one showed a heritable increase in the production of self seed. Of the 15 SLR1-mutant SRK transformed lines tested, two of these showed an increase in the amount of self-seed although only one of these did so in a consistent fashion in the progeny. Therefore, one line transformed with each of the two constructs was studied further. Twenty-five self-progeny of each of the transformed lines were analyzed and in every case where the transgene was present (62/78 for the A10 mutant SRK line), the progeny set self seed while in every case where the transgene was absent the plants were SI. Therefore, the change in phenotype to SC segregated with the transgene.

It is expected that other constructs targeting other alleles associated with the self-incompatibility phenotype could be developed and transformed into self-incompatible cultivars, using the same techniques described in the previous examples and using techniques known to those skilled in the art. It is also expected that these techniques could be used for genera other than *Brassica*.

Example 3: Phenotype of the lines transformed with the mutant SRK gene

The transformed lines were analyzed for their ability to set self-seed, the level of fertility when cross-pollinated with the W1 line and the ability of the pollen to germinate and form pollen tubes. The A10-mutant SRK transformed line had virtually the same level of seed set as the SC line Westar when it was self-pollinated (Table 1).

Seed pods were uniformly full for this line and with regard to self-fertilization, it was virtually indistinguishable from an SC line. When reciprocal crosses were done with the W1 line, pollen from W1 was able to fertilize the transgenic line. However, pollen from the transgenic line was rejected by the W1 line with virtually no seed set (see Table 1). An analysis of pollen germination and tube growth corresponded exactly with the seed set results with self-pollen and pollen from W1 both being able to germinate and their pollen tubes growing normally. In contrast, pollen from the transgenic line would not germinate on W1 stigmas. Therefore, only the stigma side of the SI phenotype was affected by the transgene, with no noticeable change in the

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pollen phenotype.

The SLR1-mutant SRK transformed line did not show a complete restoration of self-fertility. The seed pods on any individual plant were quite variable and the average seed set, while much higher than for the SI W1 line, was lower than for the SC Westar line (Table 1). Therefore, in this case there was only a partial breakdown in the SI phenotype. Crosses between this line and W1 again demonstrated that only the stigma phenotype was affected with seed only being formed when the W1 pollen was used to fertilize the transgenic line with the reciprocal cross giving no seed set (Table 1). In this case, when either self pollen or W1 pollen was germinated on stigmas from the transgenic line, only a small percentage of pollen would germinate and form pollen tubes. This supports the notion that this line is intermediate in phenotype between SC and SI lines in the stigma, with the pollen phenotype not being affected at all.

Example 4: The expression of the SLG and SRK genes in the A10-mutant SRK line

In order to determine the mechanism by which the A10-mutant 910 SRK transgene was affecting phenotype, the levels of the 910 SRK and 910-SLG transcripts were analyzed using RT-PCR and RNA blot analyses respectively (Figure 3 and 4). It is clear from these results that the 910 mutant SRK transgene is working via co-suppression with both the SLG and SRK transcripts being virtually completely absent. The receptor portion of the 910-SRK gene is 90% similar in sequence to the 910-SLG coding region and this is clearly sufficient to get the co-suppression effect.

In *Brassica napus* there is also the non-functional A-10 allele present in the b genome (Goring et al, 1992a) (the 910-allele was originally derived from *B. rapa* and is thus in the c genome). The level of A10-SLG transcript was also analyzed using RNA blot hybridization with the results shown in Figure 5. It is clear that this transcript was also present at very low levels. The 910-SRK is 85% similar in DNA sequence to the A10-SLG coding region. Therefore, the transgene co-suppresses the expression of the 910 SLG and SRK, as well as the A10 SLG which is present at the S-locus in the other genome of *B. napus*.

Example 5: Transfer of the A10-mutant SRK transgene into a line with a different functional S-allele

The *Brassica napus* line carries the A14 S-allele (Goring et al, 1992a).

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Progeny from the original transgenic line carrying the A10-mutant SRK chimeric gene was used as a pollen donor in a cross with T2. Progeny from this cross would be expected to have the transgene, the 910 S-allele present in the W1 line and the A14 S-allele found in T2 all present in a heterozygous state. This indeed turned out to be the case when these were analyzed for their presence by PCR and by their segregation into the subsequent generation. As shown in Table 1, these plants set self seed at a high rate similar to the SC line Topas and much higher than is seen for the T2 line where virtually no self-seed was set. The expression of the A14 SLG was analyzed and it was present at very low levels, as was the 910-SLG in these plants (Figure 3). The A14 SLG is 83% similar in its coding sequence to the 910-SRK. Therefore, not only can the A10-mutant SRK prevent the functioning of the 910 S-allele, but can also prevent the phenotypic expression of a different allele.

Example 6: Effect of the SLR1-mutant SRK transgene on expression of the SLG and SRK genes

The expression of the 910-SLG and 910-SRK was analyzed in the line carrying the SLR1-mutant transgene which set self seed. In this case, there was no significant decrease in the expression of these genes (see Figure 4). The mutant SRK transgene is expressed in this case (see Figure 5). Therefore, in this case, co-suppression is clearly not the mechanism involved in the breakdown of the SI phenotype. Instead, the expression of the transgene and presumably the production of the mutant 910 SRK protein must be having an effect. Thus, the kinase mutant transgene can work either by suppressing expression of the wild-type gene or through expression of the mutant SRK.

Example 7: Construction of an SRK antisense gene

A 1.6 kb portion of the 910-SRK cDNA clone between a Sall site at position 1123 and a HindIII site at the end of the gene (Goring and Rothstein, 1992) was cloned next to the regulatory regions in an orientation that would lead to the production of antisense RNA (see Figure 5). The A10-SLG, the SLR1 and the TA39 promoters were used for this purpose. These chimeric genes were transformed into the W1 line and self-compatible lines were selected as described in Example 2.

Materials and Methods

Identifying Alleles Which Confer SI Phenotype - The procedures for identifying and cloning a self-incompatibility gene are the same as those set out in Goring et al,

1992b; Goring and Rothstein, 1992.

Constructs - Once it has been confirmed that the desired sequence to include in the clone, clones are constructed in accordance with standard molecular techniques (Sambrook et al, 1989). The 910-SRK mutant gene construct was made in the following fashion. The mutation in the kinase portion of the gene changing codon 557 from a lysine to an alanine codon is described in Goring and Rothstein, 1992. This portion of the mutated SRK cDNA was joined together with the rest of the SRK cDNA to make a clone that is identical to the wild-type 910 SRK cDNA except for that it codes for an alanine instead of a lysine at codon 557. This mutated 910 SRK was then placed adjacent to the A10 SLG and SLR1 promoters in the correct orientation for the expression of the 910 mutant SRK.

The antisense genes were constructed by inserting a Sall-HindIII fragment from the 910-SRK cDNA clone (nucleotides 1123-2755- Goring and Rothstein, 1992) adjacent to the A10 SLG, the SLR1 and the TA39 promoters. The orientation of this fragment relative to the promoters was such that antisense RNA would be made once these chimeric genes were transformed into plants.

Transformation - Several methods are known in the art for transferring cloned DNA into plants. These include the use of the *Agrobacterium tumefaciens* system (Bevan et al, 1994), electroporation-facilitated DNA, treatment of protoplasts with polyethylene glycol and bombardment of cells with DNA laden microprojectiles. Each of these techniques has advantages and disadvantages. For Brassica transformation, *Agrobacterium*-mediated transformation is normally used (Moloney et al, 1989). In each of the techniques, DNA from a plasmid is genetically engineered such that it contains not only the gene of interest, but also selectable and screenable marker genes. A selectable marker gene is used to select only those cells that have integrated copies of the plasmid (the construction is such that the gene of interest and the selectable and screenable genes are transferred as a unit). The screenable gene provides another check for the successful culturing of only those cells carrying the genes of interest. A commonly used selectable marker gene is neomycin phosphotransferase II (NPT II). This gene conveys resistance to kanamycin, a compound that can be added directly to the growth media on which the cells grow. Plant cells are normally susceptible to kanamycin and, as a result, die. The presence

of the NPT II gene overcomes the effects of the kanamycin and each cell with this gene remains viable. Another selectable marker gene which can be employed in the practice of this invention is the gene which confers resistance to the herbicide glufosinate (Basta). A screenable gene commonly used is the β -glucuronidase gene (GUS). The presence of this gene is characterized using a histochemical reaction in which a sample of putatively transformed cells is treated with a GUS assay solution. After an appropriate incubation, the cells containing the GUS gene turn blue. Preferably the plasmid will contain both selectable and screenable marker genes.

The plasmid containing one or more of these genes is introduced into plant cells by any of the previously mentioned techniques. If the marker gene is a selectable gene, only those cells that have incorporated the DNA package survive under selection with the appropriate phytotoxic agent. Once the appropriate cells are identified and propagated, plants are regenerated. Progeny from the transformed plants must be tested to insure that the DNA package has been successfully integrated into the plant genome.

The gene engineered in the foregoing manner is introduced into the plant through known transformation techniques. The appropriate plant types are selected. In normal plant biotechnology, once the desired genotype is identified following transformation and regeneration, the plants are selfed to recover that genotype.

Generating Inbred Plants Using Construct - The construct containing the transgene can be introduced into *Brassica* inbred lines by repeated backcrosses of the *Brassica* plant. For instance, the resulting seed may be planted in accordance with conventional *Brassica*-growing procedures and following self-pollination *Brassica* seed are formed thereon. Again, the resulting seed may be planted and following self-pollination, next generation *Brassica* seed are formed thereon. The initial development of the line (the first couple of generations of the *Brassica* seed) preferably is carried out in a greenhouse in which the pollination is carefully controlled and monitored. This way, the desirable characteristics of the *Brassica* seed for subsequent use in field trials can be verified. In subsequent generations, planting of the *Brassica* seed preferably is carried out in field trials. Additional *Brassica* seed which are formed as a result of such self-pollination in the present or a subsequent generation are harvested and are subjected to analysis for the desired trait, using

techniques known to those skilled in the art.

Generating Hybrid Plants Using Construct - This invention enables a plant breeder to incorporate the desirable qualities of a female self-incompatible parent and a male self-compatible parent into a commercially desirable *Brassica* hybrid variety.

5 *Brassica* plants may be regenerated from the parents of this invention using known techniques. For instance, the resulting seed may be planted in accordance with conventional *Brassica*-growing procedures and following cross-pollination *Brassica* seed are formed on the female parent. The planting of the *Brassica* seed may be carried out in a greenhouse or in field trials. Additional *Brassica* seed which are
10 formed as a result of such cross-pollination in the present generation are harvested and are subjected to analysis for the desired trait. *Brassica napus*, *Brassica campestris*, and *Brassica juncea* are *Brassica* species which could be used in this invention using known techniques.

The hybrid may be a single-cross hybrid, a double-cross hybrid, a three-way
15 cross hybrid, a composite hybrid, a blended hybrid, a fully restored hybrid and any other hybrid or synthetic variety that is known to those skilled in the art, using this invention.

Generating Plants from Plant Parts - *Brassica* plants may be regenerated from the plant parts of the *Brassica* plant of this invention using known techniques. For
20 instance, the resulting seed may be planted in accordance with conventional *Brassica*-growing procedures and following self-pollination *Brassica* seed are formed thereon. Alternatively, doubled haploid plantlets may be extracted to immediately form homozygous plants.

Breeding Techniques - It has been found that the combination of desired traits
25 described herein, once established, can be transferred into other plants within the same *Brassica napus*, *Brassica campestris*, or *Brassica juncea* species by conventional plant breeding techniques involving cross-pollination and selection of the progeny.

Also, once established the desired traits can be transferred between the *napus*,
30 *campestris*, and *juncea* species using the same conventional plant breeding techniques involving pollen transfer and selection. The transfer of traits between *Brassica* species, such as *napus* and *campestris*, by standard plant breeding

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techniques is already well documented in the technical literature. (See, for instance, Tsunada et al., 1980).

Stand of Plants -The improved *Brassica* plant of the present invention is capable of production in the field under conventional *Brassica* growing conditions that are commonly utilized during seed production on a commercial scale. Such seed of *Brassica* exhibits satisfactory agronomic characteristics and is capable upon self-pollination of forming seed and meal providing satisfactory agronomic characteristics. For the purposes of the present invention, "satisfactory agronomic characteristics" is defined as the ability to yield an seed harvest under standard field growing conditions meet the standards required for registration of canola varieties (suitable for commercial use).

All publications, patents and patent applications, including the priority application U.S. Serial No. 60/037,719 are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

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Average Seed Set per Pod

Westar (Selfed)	W1 (Selfed)	W1 SRK Mutant (Selfed)	W1 ♀ x SRK Mutant	SRK Mutant x W1 ♂
29	0.2	19	0.1	22

Topas (Selfed)	T2 (Selfed)	T2 / W1 SRK Mutant (Selfed)
> 25	< 1.0	24

TABLE 1

We claim:

1. A cell of a self-compatible hybrid plant, the parents of which are a self-incompatible female parent and a male parent, and the nuclear genome of which contains, stably integrated therein, a vector, comprising
 - a) DNA sequence which, when expressed in a cell of the plant, imparts phenotype for self-compatibility to the plant, and which is selected from a group consisting of
 - i) a mutant of a DNA sequence which imparts self-incompatibility to the plant,
 - ii) a DNA sequence which expresses an RNA sequence which is antisense to an RNA sequence encoding a protein or polypeptide which imparts self-incompatibility to the plant, and
 - iii) a DNA sequence which is antisense to a DNA sequence which imparts self-incompatibility to the plant.
 - b) a promoter capable of directing the expression of the DNA sequence in the cell.
2. The cell of claim 1, wherein the plant is from the genera *Brassica*.
3. The cell of claim 2, wherein the species of plant is selected from a group consisting of *Brassica napus*, *Brassica campestris* and *Brassica juncea*.
4. The cell of claim 1, wherein the species of the plant is not from the genera *Brassica* but has a S-receptor kinase transgene to knock out self-incompatibility.
5. The cell of claim 1, wherein the cell is selected from a group consisting of a stigma cell and a pollen cell.
6. The cell of claim 1, wherein the promoter is selected from a group consisting of an A10 promoter, an SLR1 promoter and a TA39 promoter.
7. A cell of a self-incompatible plant, the nuclear genome of which contains, stably integrated therein, a vector, comprising
 - a) a mutant DNA sequence which, when expressed in a cell of the progeny of the plant, imparts a phenotype for self-compatibility to the plant, and
 - b) a promoter capable of directing the expression of the DNA sequence in the cell.

8. A vector, comprising
 - a) a mutant DNA sequence which, when expressed in a cell of the progeny of a plant, imparts a phenotype for self-compatibility to the plant, and
 - b) a promoter capable of directing the expression of the DNA sequence in the cell.
9. A plant, comprising the cell of any of claims 1-6.
10. A plant transformed with the vector of claim 8.
11. The plant of any of claims 9-10, comprising a *Brassica* plant.
12. The plant of claim 11, wherein the *Brassica* plant is selected from a group consisting of a hybrid and an inbred.
13. A DNA sequence selected from a group consisting of
 - a) a mutant of a DNA sequence which imparts self-incompatibility to the plant,
 - b) a DNA sequence which expresses an RNA sequence which is antisense to an RNA sequence encoding a protein or polypeptide which imparts self-incompatibility to the plant, and
 - c) a DNA sequence which is antisense to a DNA sequence which imparts self-incompatibility to the plant.
14. Seed of the plants of any of claims 9-12.
15. A method of breeding plants by conferring a self-compatible phenotype on progeny of a homozygous self-incompatible plant and a homozygous self-compatible plant, comprising transforming the vector of claim 8 into a self-incompatible plant, plant tissue or plant protoplast and expressing a phenotype for self-compatibility in the progeny.
16. The method of claim 15, wherein the self-incompatible plant is of the genus *Brassica*.

17. A method for producing self-compatible hybrid *Brassica* seed from a homozygous self-compatible male parent *Brassica* plant and a homozygous self-incompatible female parent *Brassica* plant, comprising
 - a) transforming the native nuclear genome of a homozygous self-incompatible female parent *Brassica* plant with the vector of claim 8;
 - b) crossing the self-incompatible female plant with the male parent to produce self-compatible hybrid seed ; and
 - c) harvesting the self-compatible hybrid seed.
18. The method of claim 17, comprising an additional step of inbreeding the self-incompatible female parent *Brassica* plant before crossing the self-incompatible plant with the male parent.
19. A method of producing hybrid seed, comprising the steps of planting, in cross pollinating juxtaposition, a first seed from a homozygous self-compatible male parent line and a second seed from a homozygous self-incompatible female line having the vector of claim 8;
 - a) growing the seed to mature plants;
 - b) cross pollinating the self-incompatible female plant with pollen from the self-compatible male plant to produce self-compatible hybrid seed; and
 - c) harvesting the self-compatible hybrid seed.
20. A part of a plant of any of claims 9-12.
21. The plant part of claim 20, wherein the part is selected from a group consisting of nucleic acid sequences, tissue, cells, pollen, ovules, roots, leaves, seed, microspores, vegetative parts, whether mature or embryonic.
22. The plant part of claim 21, wherein the nucleic acid sequences are selected from a group consisting of RNA, mRNA, DNA, cDNA.
23. The use of a *Brassica* plant of claim 11 for the breeding of a *Brassica* line.
24. The use of a *Brassica* plant of claim 12 for the breeding of a *Brassica* line.
25. The use of claim 23 or 24, wherein the breeding is selected from a group consisting of isolation and transformation, conventional breeding, pedigree breeding, crossing, self-pollination, haploidy, single seed descent and backcrossing.

Mutant SRK Constructs

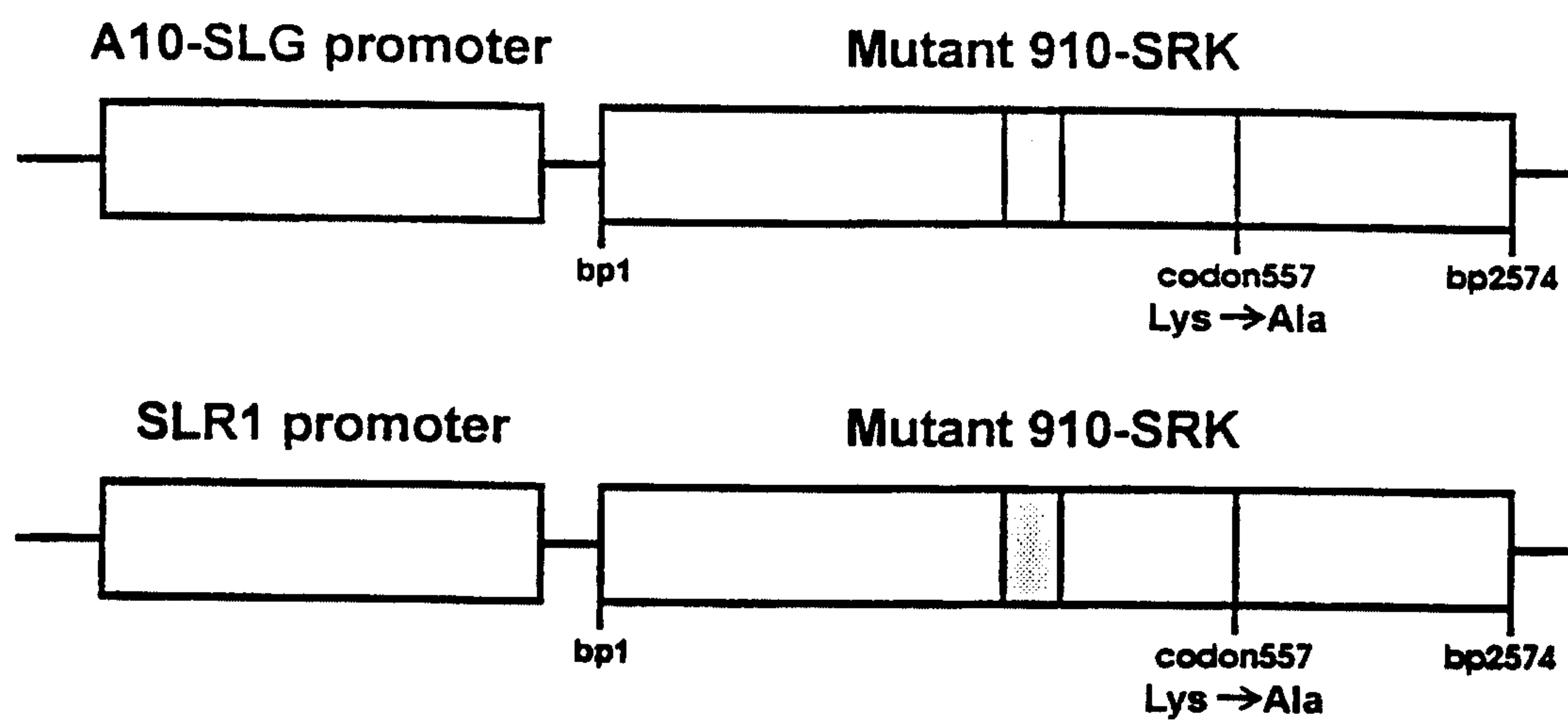


Figure 1

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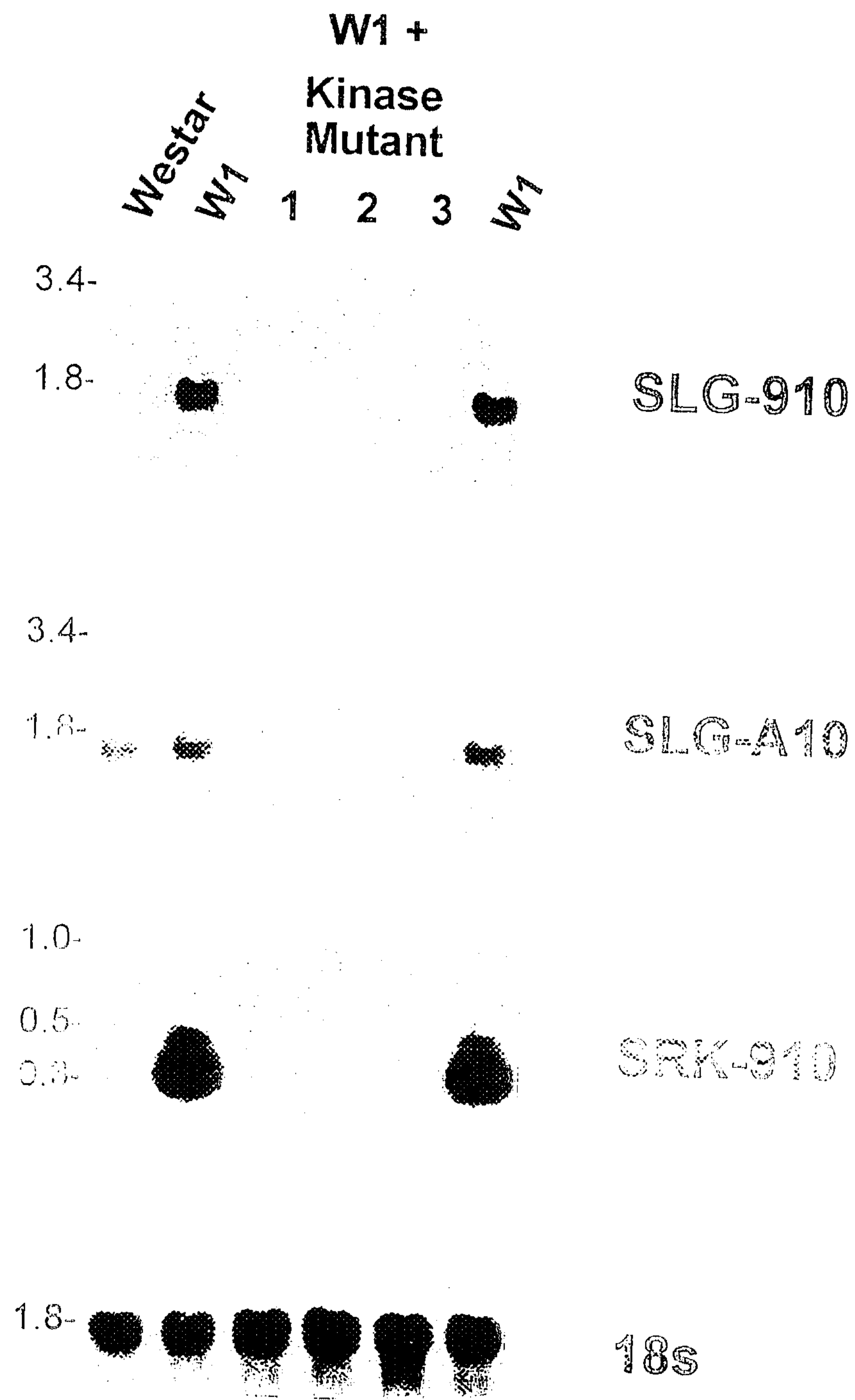


FIGURE 2

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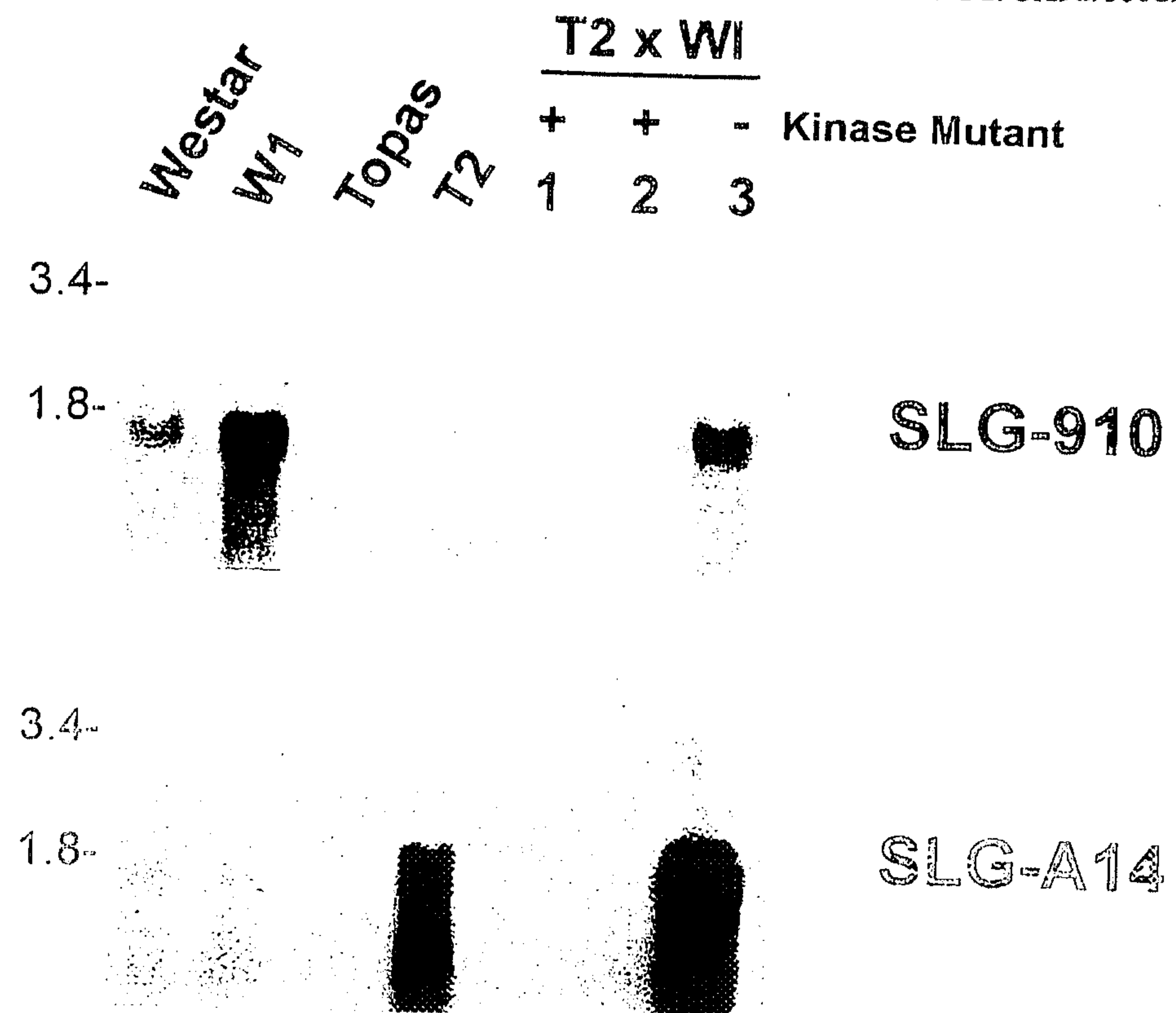


FIGURE 3

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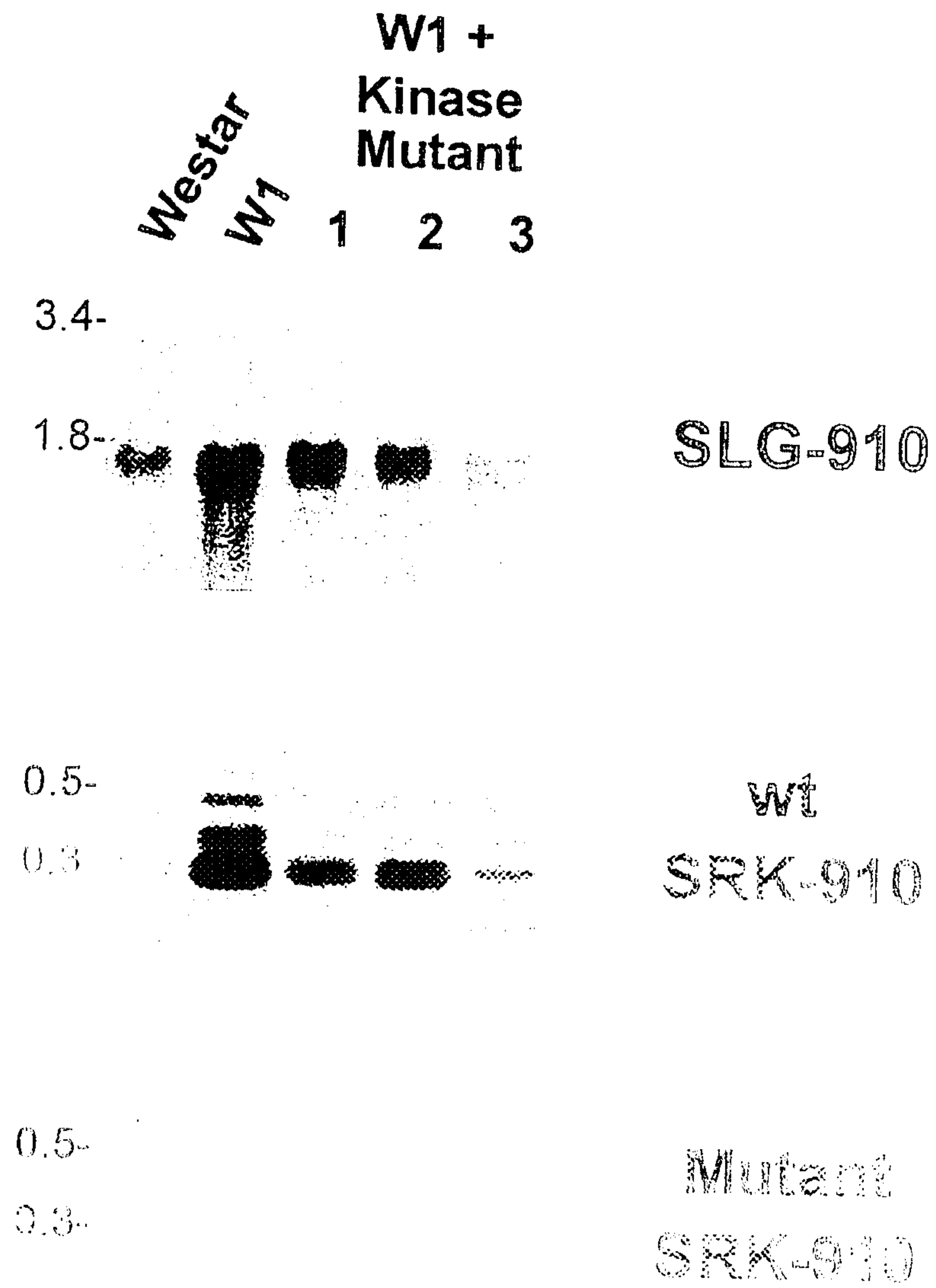


FIGURE 4

Antisense SRK Constructs

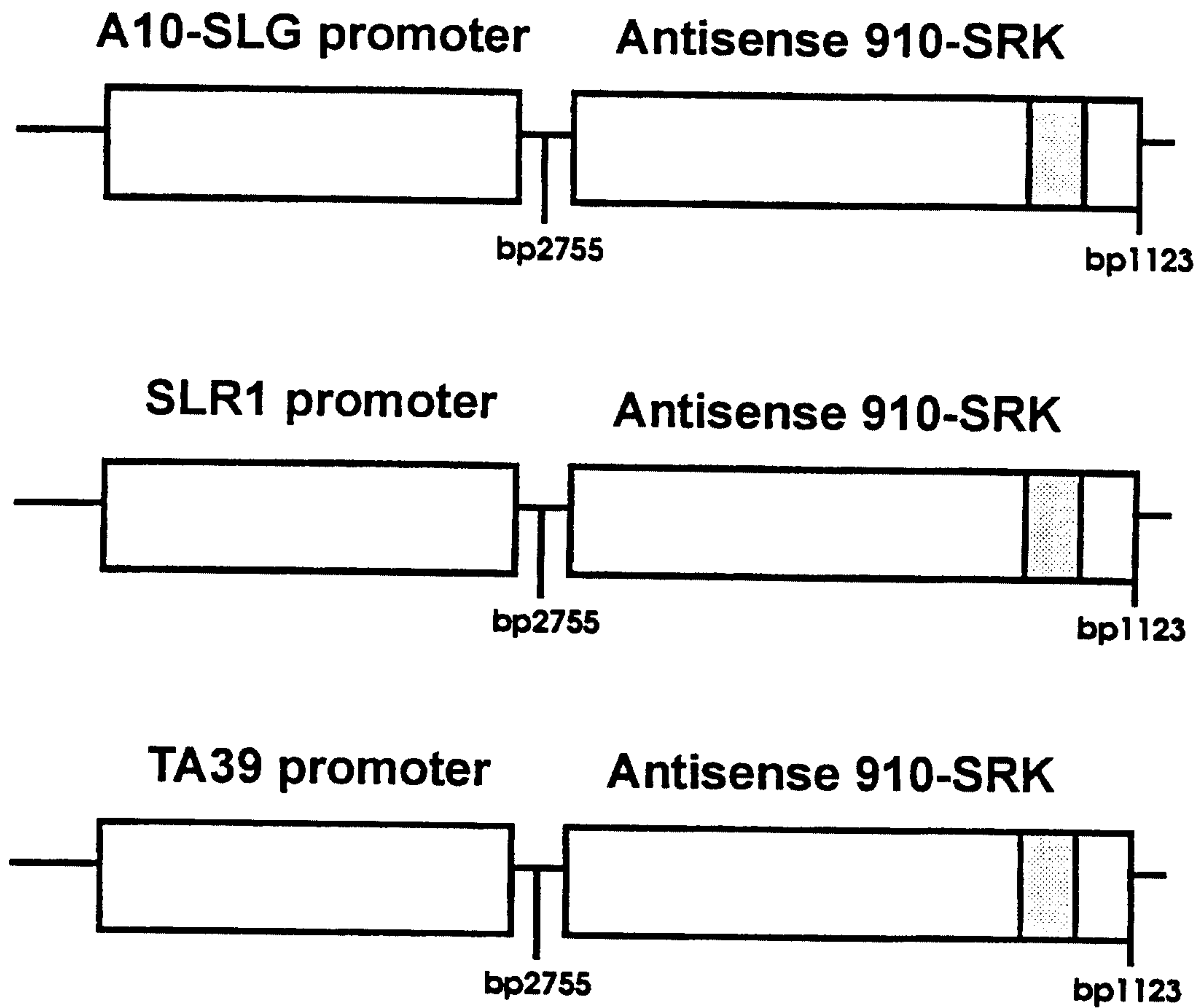


FIGURE 5

FIG. 6A

M K G V R K T Y D S S Y T L S F L L V F F V M F L F H P A L S I H I N T L S S T	40
ATGAAAGGAGTAAAGAAACCTACGATAGTCTTACACTTTATCCCTTCTGCTCGCTTTTTCGTCATGTTCTATTTATCTTCCTCCCTTCGATCCATATCAACACTTTTGTCGTCACCA	120
E S L T I S H N R T L V S P G N V F E L G F F R T T S S S R W Y L G I W Y K N L	80
GAATCTCITACAACTCAACACACAGAACACTTGTGCTCCAGGTAAATGTCTTCGAGCTCGGCTTCITTAGAACCCACCTCAAGTTCCTCGTTGGTAICTCGGGATATGGTACACAGAAATTG	240
P Y K T Y V W V A N R D N P L S D S I G T L K I S H N H L V L L D H S N K S V W	120
CCCTATAAAACCTATGTTTGGGTTGCAACACAGAGATAACCCCTCTCTCCGATTCCATTGGTAGCCTCAAAATCTCCACATGAACTTCCTCCCTCCCTGACCCACCTCAATATAATCTCIGTTTGG	360
S T N L T R G N E R S P V V A E L L E N G N F V I R Y S N N N A S G F L W Q S	160
TCGACGAATCTGACIAGAGGAATGAGAGATCTCCGGTGGTGGCAGAGCTTCTGGAGAACGGAACTTCGTCATTCGATACTCCAAATPACACACACGCAAGIGGATTCCTTGTCGCAAACT	480
F D F P T D T L L P E H K L G Y D R K K G L N R F L T A W R N S D D P S S G E I	200
TTCGATTCCCTACAGATACTTTGCTTCCAGAGATGAACCTAGGCTACGACCGCAAAAGGGCTGACAGATTCCTTACAGCATGGAGAAATTCAGATGATCCCTCAAGCGGGGAAATC	600
S Y Q L D T Q R G H P E F Y L L K N G V R G Y R S G P W N G V R F N G I P E D Q	240
TCGTACCAACTAGACACTCAAGAGAGGAATGCCCTGAGTTTATCTATTGPAAAACGGCGTACGAGGCTACCGGAGTGCTCCATGGAAITGGAGTCCGATTTAATGGCATACCCAGAGGACCAA	720
K L S Y H V Y N F T D N S E E A A Y T F R M T D K S I Y S R L I I S H D E Y L A	280
AAGTTGAGTTACATGGTGTAACACTTCACAGATNAATAGTGAGGAGGCTGCTTATACATTTTCGAATGACCGACACAGAGCATCTACTCGAGATTGATAATAAGTAACGATGAGTATTTGGCG	840
R L T F T P T S W E H N L F H T S P E E P E C D V Y K T C G S Y A Y C D V N T S	320
CGACTAACGTTCACTCCGACATCATGGGAATGGNACTTGTTCTCGACTTCACCAGAGGAGCCGGAGTGCGGATGIGTACACAGACTTGTGGGTCTTATGCTTACTGTGACGTGACGTGAACACATCA	960
P V C N C I Q G F K P F N M Q Q W E L R V W A G G C E I R R T R L S C N G D G F T	360
CCAGTGTGTAACCTGATCCAAAGGTTTCAAGCCCTTCRAATATGCACAGCAGTGGGAACCTGAGAGICTGGGCAGGTGGGTGTATAAGGAGGACCGCGCTTAGCTGCRAATGGAGATGGTTTACC	1080
R H K N H K L P E T T H A I V D R S I G R K E C K R C L S D C N C T A F A H A	400
AGGATGAPAAATATGAAGTTGCCAGAAACTACGATGGCTATGTGACCCGACGTATGTGTCGGAAAGAATGTAAAGAAGAGGTGCCCTTAGCGATTGTAAATGTACCGCGTTTGCAAAATGCG	1200
D I R N G G S G C V I W T G E L E D I R N Y F D D G Q D L Y V R I A A A D L V F	440
GATATCCGGATGGTGGGTGCTGATTTGGACAGGAGAGCTTGAGGATATCCGGATTACTTTGATGACGGTCAAGATCTTTATGTACAGATTGGCTGCGCTGATCICGTTTAA	1320
K R N A N G K T I A L I V G V C V L L L H I H F C L H K R K Q K R A K T T A T S	480
NAGAGAAACCGGAATGGGAACCTATAGGTTGATTTGGAGTTTGGTTCCTTCTTATGATCACTGTTCTGCTCTTGGAAAGGAAACAAAGCGAGCAAAACAACTGCAACATCT	1440

FIG. 6B

I V N R Q R N Q D L L L H N G H I L S S K R Q L P I E N K T E E L E L P L I E L E 520
 ATTGTAAATCGACAGAGAAACCAAGATTGCTAATGACGGGATGATACATACTATCAAGCAAGAGACAGTTGCCCTATAGAGAACAAACIGAGGAATTGGAACTTCCATTGATAGATTGGAA 1560
 A V V K A T E H F S N C N K L G Q G G F G I V Y K G R I L D G Q E I A V A R I S 560
 GCTGTGTCAAAGCCACCGAAATTTCTCCRAATTGTAAACPAACCTCGGACCAAGGTGGTTTCGGTATTGTTTACRAAGGTAGATTACTTGTGATGGGCAAGAAATTCGGTAGCAAGGCTATCA 1680
 K T S V Q G T G E F H N E V R L I A R L Q H I N L V R I L G C C I E A D E K H L 600
 AAAACGTCCGTTCAAGGGACTGGTGAGTTTATGATGAGGTGAGATTGATCGGAGGCTTCAGCATATARAACCTTGTCGGAATTCITGGCTGTTGCAATGGAGGCAGACGAGAGATGCTG 1800
 V Y E Y L E N L S L D S Y L F G N K R S S T L N H K D K F H I T N G V A R G L L 640
 GTATATGAGTATTAGAAATTTAAGCCCTCGATTCTTATCTTCGGAAATTAACGAAGCTCTACGTTTAAATTGGRAAGGACAGATTCAACATTACCAATGGTGTGCTCGAGGACCTTTA 1920
 Y L H Q D S R F R I I H R D H K V S N I L L D K N H T P K I S D F G H A R I F A 680
 TATCTTCATCAAGACTCAGCGTTTAGGATAATCCACAGAGATATGAAGTAAGTACATTTTGGTTCATTAATAATATGACACCAAGATCTCGGATTTTGGGATGGCCAGAAATCTTTGCA 2040
 R D E T E A N T R K V V G T Y G Y H S P E Y A H D G V F S E K S D V F S F G V I 720
 AGGACGAGACTGAAGCTAACACAGGAAGGTGGTGGTACATGCTCCGGAGTACGAATGGGATGGGTAATCTCGGAAATTCAGATGTTTTCAGTTTGGAGTCAAT 2160
 V L E I V S G K R N R G F Y N L N H E N N L L S Y V W S H W T E G R A L E I V D 760
 GTTCTTGAAATTGTAGTGGAAAGGACAGAGGATTCTACAACTTGAACCAAGAAAGTCTTAAGCTATGTATGGAGTCACTGGACGGAGGAGAGCGCTAGAAATTTGTGAT 2280
 P V I V D S L S L P A T F Q P K E V L K C I Q I G L L C V Q E R A E H R P T M 800
 CCAGTCATCGTAGATTTCATTGTCATCATACCAGCAACCTTTCAACCAAGAAAGTCTTAAGTGCATACAAATTTGGTCTCTGTGTTCAGGAACGTGCAGAGCATAGACCAAGGATG 2400
 S S V V W H L G S E A T E I P Q P T P G Y S L G R S P Y E N N P S S S R H C D 840
 TCGTCCGTGGTTTGGATGCTTGGAGTGAAGCAACAGAGATTCTCAGCCCTACACCGCCAGGTTATTCCTCGGAAGAAGTCCCTTATGAATAATATCCTTCATCAAGTAGACATTGGGAC 2520
 D D E S W T V N Q Y T C S D I D A R 2640
 GACGACGAATCCTGGACGGTGAACCAAGTACACCTGCTCAGACATCGATGCCCGGTAGTACGPAATCCGTTGAGAAAGTTCAGATAATTAACCTATTGGGGTGACCGGATATTATAAGTGAA 2640
 AGAATAAATTCATATAGTTTAAAGTTTATTGATAACCAAAATCTTGTATTTTCTGATGAATGTTTAAAGTTATTATTC 2749

1 AACAATATAA TAACAAAAA AAAATAGAGA M K G Y R K T Y O S S Y T L S F L L Y F F V M F L F H P A L
TTGTTATATT ATTGTTTTT TTTATCTCT CTACTTTCTT CATTCCTTTT GGATGCTATC AAGAATGTGA AATAGGAAGA ACGAGCAGAA AAAGCAGTAC AAAGATAAG TAGAAGCGGA

121 S I H I N T L S S T E S L T I S N N R T L Y S P G N Y F E L G F F R T T S S S R
TTGATCCAT ATCAACACTT TGTCGTCTAC AGAATCTCTT ACAATCTCAA ACAACAGAAC ACTTGTGTCT CCAGGTAATG TCTTCGAGCT CGGCTTCTTT AGAACCACTT CAAGTTCTCTG
AAGCTAGGTA TAGTTGTGAA ACAGCAGATG TCTTAGAGAA TGTTAGAGTT TGTGTCTTG TGAACACAGA GGTCCTATTAC AGAAGCTCGA GCGGAAGAAA TCTTGGTGGG GTTCAAGAGC

241 V Y L G I W Y K N L P Y K T Y V V V V A N R D N P L S D S I G T L K I S N M N L Y
TTGGTATCTC GGGATATGCT ACAAGAATTT GCGCTATAAA ACCTATGTTT GGGTTGCAAA CAGAGATAAC CTCTCTCTCG ATTCCATTGG TACGCTCAAA ATCTCCAACA TGAACCTTGT
AACCATAGAG CCTATACCA TGTCTTAAA CCGGATATTT TGGATACAAA CCCAACGTTT GTCTCTATTG GGAGAGAGGC TAAGGTAACC ATGCGAGTTT TAGAGTTGT ACTTGAACA

361 L L O H S N K S Y V S T N L T R G N E R S P V V A E L L E N G N F Y I R Y S N H
CCTCTCTGAC CACTCTAATA AATCTGTTTG GTGACGGAAT CTGACTAGAG GAAATGAGAG ATCTCTGGTG GTGGCAGAGC TTCTGGAGAA CCGAACTTC GTCTTCGAT ACTCCAATAA
GGAGGAGCTG GTGAGATTAT TTAGACAAAC CAGCTGCTTA GACTGATCTC CTCTACTCTC TAGAGGCCAC CACGCTCTCG AAGAGCTCTT GCTTTGAGG CAGTAAGCTA TGAAGTTATT

481 N H A S G F L Y O S F D F P T O T L L P E M K L G Y D R K K G L N R F L T A V R
CAACAACGCA AGTGGATTCT TGTGGCAAG TTTGATTTC CTACAGATA CTCTGCTTCC AGAGATGAAA CTAGGCTAGC ACCTGCTTCTT AGGCTGAAAC AGATTCTTA CAGCATGAG
GTTGTTGCTT TCACCTAAGA ACACCTTTT AAAGCTAAG GGTATCTAT GAAACGAAG TCTCTACTTT GATCCGATGC TGGCTTTT TCCGACTTG TCTAAGCTA GTCTAGCTC

601 N S D O P S S G E I S Y O L O Y O R G M P E F Y L L K N G V R G Y R S G P W N G
AAATTCAGAT GATCCCTCAA GCGGGGAAAT CTCTGACCAA CTAGACTCTC AAAGAGGAAT GCCTGATTTT TATCTATTGA AAAACGGCTT ACAGGCTAC CCGAGTGGT CATGATG
TTAAAGCTA CTAGGAGTT CCGCTCTTA GAGCATGTT GATCTGTGAG TTTCTCTTA GCGACTCAAA ATAGATAACT TTTTGGCGA TGCTCCGATG GCTCACCAG GTACTTACC

721 V R F N G I P E D O K L S Y M V Y N F T O N S E E A A Y T F R M T D K S I Y S R
AGTCCGATT TATGGCATAC CAGAGGACCA AAAGTTGAGT TACATGTTGT ACACTTCTAC AGATAATAGT GAGGAGGCTG CTTATACATT TCGAATGACC GACAAGACCA TCTACTGAG
TCAGGCTAAA TTACCTATG GTCTCTGCTT TTTCAACTCA ATGTACCACA TGTGAAGTG TCTATTATCA CTCTCTGAC GAATATGTA AGCTTACTGG CTGTTCTCT AGATGAGCTC

841 L I I S N O E Y L A R L T F T P T S W E W N L F M T S P E E P E C D V Y K T C G
ATTGATAATA AGTAACGATG AGTATTTGGC GCGACTAAGC TTCCTCTGCA CATCATGGA ATGGAATCTG TTCTGGACTT CACGAGAGGA GCGGAGTGC GATGTTTACA AGCTTTGAG
TAAGTATTAT TCATTGCTAC TCATAAACCG CCGTGATTGC AAGTGAGGCT GTAGTACCT TACCTTGAAC AAGAGCTGAA GTGGTCTCT CCGCTCAGG CTACACATGT TCTAACACC

961 S Y A Y C D V N T S P V C N C I O G F K P F N M O Q W E L R Y N A G G C I R R T
GTCTTATGCT TACTGTGAGC TGAACACATC ACCAGTGTGT AACTGTATCC AAGGTTTCAA GCGCTTCAAT ATGAGCAGT GGAAGCTGAG AGTCTGGCA GGTGGTGT TAAGAGGAC
CAGAATACGA ATGACTGTC ACTTGTGTAG TGGTCACACA TTGACATAGG TTCCAAGTT CCGGAAGTTA TACCTGCTCA CCGTGTACTC TCAGACCGCT CCACCCACAT ATCTCTCTG

1081 R L S C N G D G F T R M K N M E L P E T T M A I V D R S I G R R E C K R R C L S
GCGGCTTAGC TGAATGGAG ATGTTTTC CAGGATGAAA AATATAGAT TGCAGAGAAC TACATGCTT ATTCTGACC GCAATATTGG TCGGAGAGAA TGTAGAGAA GTGCTTAG
CGCGAATCG ACGTTACTC TACCAAAATG GTCTACTTT TTATCTTCA ACGGTTTGT ATGCTACCA TAACAGCTGG CGTCATAACC AGCTTTCTT ACATTCTCT CCACGAAATC

1201 D C N C Y A F A N A O I R N G G S G C Y I M T G E L E D I R N Y F D O G O D L Y
CGATTGTAAT TGTACCGCT TTGCAATGC GGATATCCGG AATGTTGGT CCGTTGTGT GATTGGAACA GGAGAGCTTG AGGATATCC GAATYACTTT GATGAGGTC AATCTTTA
GCTAACATTA ACATGCGCA AAGCTTTACG CCTATAGGCC TTACACCCA GCGCAACACA CTAAAGCTGT CCTCTCGAAC TCTATAGGC CTATAGGAA CTACTGCCAG TTCTAAGAT

1321 V R L A A A D L V K K R N A N G K T I A L I V G V C V L L L M E N F C L W E R R
TGTGATTTG GCTGCGCTG ATCTCTTAA AAAGAGAAAC GCGAATGGGA AAACCATAGC GTTATTTGT GGAATTTGT TTCTCTCTT TATGATCATG TTCTGCTCTT GGAAGAGAA
ACATCTAAC CAGCGGCAAG TAGAGCAAT TTTCTCTTG CCGTTACCT TTTGATCTG CAACTAACAA CCTCAACAC AAGAGCAAGA ATACTAGTAC AAGAGCAAGA CCTTTCTCT

1441 Q K R A K T T A T S I V N R O R N O D L L M N G M I L S S K R O L P I E N K T E
ACAAAAGCA GCAAAAACAA CTGCAACATC TATTGTAAAT CAGAGAGAA ACCAAGATT GCTAATBAAC GGAATGATAC TATCAAGCAA GAGACATTT CTTATAGAA ACAAACTGA
TGTCTTCTT CTTTTTCTT GAGTTGTAG ATAACATTA GCTGTCTCT TGTCTCTAAA CATTACTTG CCGTACTATG ATAGTTCTT CTCTGCAAC GATATCTCT TTTTTACT

1561 E L E L P L I E L E A V V K A T E N F S N C N K L G O G G F G I V Y K G R L L D
GGAATTTGAA CTCTCATTA TAGAGTTGGA AGCTTTTGC AAAGCCACCG AAAATTTCTC CAATTGTAA AAACCTGGAC AAGTTGTTT CCGTATTCTT TACAAGGTA GATTACTTA
CCTTAACCTT GAAGGTAACT ATCTCAACCT TCGACAACAG TTTCTGTTGG TTTTAAAGAG GTTAACATTG TTGAGGCTG TTCCACCAA GCGTAACAA ATGTTCCAT CTATBACT

1681 G O E I A V A R L S K T S V O G T G E F M N E Y R L I A R L O N I N L V R I L G
TGGCAAGAA ATGCGGTA GAGGCTATC AAAACGTCG GTTCAAGGA CTGTTGAGT TATGAATGAG GTGAGATTGA TCGGAGGCT TCAGCATATA AACCTTGTG GAATTTCTG
ACCGTTCTT TAAGGCTAG GTTCCGATG TTTTTCAGC CAAGTTCCCT GAGCACTCAA ATACTACTC CACTCTAAT AGGCTCTGGA AGTCTATAT TTGGAACAG GTTAAGAAC

1801 C C I E A O E R M L Y Y E Y L E N L S L O S Y L F G N E R S S T L N V K D R F N
CTGTTGCAAT GAGGAGAGC AGAAGATGCT GGTATATGAG TATTTAGAAA ATTTAAGCT CCGATTCTAT CTCTCTGGA ATAAAGCAAG CTCTAGCTA AATGGAAGC ACATCTCAA
GACAACGTAA CTCTGTCTG TCTTCTAGG CCAATATCTC ATAAATCTTT TAAATTCGGA GCTAAGATA GAGAAGCCTT TATTTGCTT GAGATGCAAT TTAACTTCT TGTCTAAT

1921 I T N G V A R G L L Y L H O D S R F R I I N R O N K Y S N I L L O K N H T P E I
CATTACCAAT GGTGTTCTC GAGGACTTTT ATATCTTCT ATAGACTCAC GGTTAGGAT AATCCACAGA GATATGAAAG TAAGTAACAT TTTGCTTGT AAAATATGA CACCAAGAT
GTAATGTTA CCAAGAGAG CTCTGAAAA TATAGAAGTA GTTCTGAGTG CCAATCTCTA TTAGGTTCT CTATCTTCT ATTCATTGA ATTCATTGA TTTTATACT GTGTTTCTA

2041 S D F G M A R I F A R O E T E A N T R K Y V G T Y G Y M S P E Y A N O G V F S E
CTGCAATTTT GGAATGCGA GAATCTTTC AAGGAGGAG ACTGAAGTA ACACAAGAA GGTGCTCGA ACTTACGCT ATATCTCTC CAGTACGCT CCGTATGCT TACCTACCC ATAAAGCTT
GAGCTAAAA CCTACCGT CTAGAGAGG TTCTGCTC TGTGTTCTT CCAAGAGCT TGAATGCGA TGTGCTCTT CCAAGAGCT TGAATGCGA TGTGCTCTT CCAAGAGCT TGAATGCGA TGTGCTCTT

2161 K S D V F S F G V I Y L E I V S G K R N R G F Y N L N N E N H L L S Y V W S N H
AAATCAGAT GTTTTCAAT TTGAGTCTT TGTCTTGA ATGTTTGTG GAAAAAGAA CAGGATCTC TACAATCTG ACCAGCAAAA CAATCTCTA AGCTATGAT GAGTCACTG
TTTTAGCTA CAAAAGTCAA AACCTCAGTA ACAAGAACTT TAAATCTC CTTTTCTT GTCTCTAA ATGTTGAAT TGTGCTTT GTTAGAAT TCGATACATA CCGTCTGAC

2281 T E G R A L E I V D P V I V O S L S S L P A T F O P K E V L K C I O I G L L C Y
GAGGAGGGA AGAGGCTAG AAATTTTGA TCCAGTCTC GTAGATTCT TGTCTCTT ACCAGCAACC TTCTCAACAA AAGAAATCT AAAATGATA CAAATGCTC TCTTGTGT
CTGCTCTCT TCTGCGATC TTTAAGAACT AGGTCAGTAG CATCTAAGTA ACAGTAAAG TGGTCTGTG AAAGTTGCTT TTCTCAAGA TTTTACGAT GTTTAAGCA AGAGACACA

2401 Q E R A E H R P T M S S V V W M L G S E A T E I P O P T P P G Y S L G R S P Y E
TCAAGACGT GCAGAGCATA GACCAAGCAT GTCTGCTGT GTTTGATGC TTGGAAGTGA AGCAACAGAG ATCTCTCAGC CTACACCGCC AGGTTATCTC CTGGAAGAA GTCTTATGA
AGTTCTTGA GTCTCTGAT CTGTTGCTA CAGCAGGAC CAAAGCTAG AACCTTCACT TCGTTGTCT TAAGGAGTGB GATGTTGCG TCAATAAGG GAGCTTCTT CAGGATACT

2521 N H P S S S R H C D D O E S M T V N O Y T C S O I D A R O
AAATATCTT TCATCAAGTA GACATTGCGA CGAGCAGGAA TCCTGAGCGG TGAACAGTA CACCTGCTCA GACATCGAT CCGGCTAGTA CGAAATCTCT TGAAGAAAT CAGATAATTA
TTTATTAGGA AGTATCTCAT CTGTAAGCT GCTGCTGCTT AGGAGCTGCC ACTTGTCTAT GTGAGGAGT CTGTAGCTAC GGGCAATCAT GCTTTAGCA ACTTTTCAA GTCTATTAT

2641 ACTATTGGGG TGAGCGGATA TTATAAGTGA AAGAAAAATA AATTCAATA GTTAAGTTTG TTATTGATA ACCAAATCTT GTTATTCTT GGTGTTGTG TCATATTCTT TTTTCTAAT
TGATAACCC ACTGGCTAT AATATCTCT TCTTTTATT TTAAGTTAT CAATTEAAG AATAAGTAT TGGTTAGAA CAATAAGGA CCAACACAG AGTATAAGCA AAAAGCTTA

2761 GAATGTTAAA GTTATTATTC
CTTACAATTT CAATAAAG

FIGURE 5C

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AMENDED SHEET