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(71) Applicant *(for all designated States except US)*: **ZENECA LIMITED** [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HOLT, Charles, David** [GB/GB]; Zeneca Agrochemicals, Jealott's Hill International Research Centre, Bracknell RG42 6YA (GB). **WHITE, Andrew, John** [GB/GB]; Unilever Research, Colworth House, Sharnbrook, Bedfordshire MK44 1LQ (GB). **MICHAEL, Anthony, John** [GB/GB]; Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA (GB). **OSBORN, Rupert, William** [GB/GB]; Plant Bioscience Limited, Norwich Research Park, Norwich NR4 7UH (GB).

(74) Agents: **GAAL, Jozsef, Christopher** et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Interantional Research Centre, P.O. Box 3538, Bracknell RG42 6YA (GB).

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(54) Title: HERBICIDE RESISTANT PLANTS AND METHODS FOR THE PRODUCTION THEREOF

(57) Abstract: The present invention relates to methods of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a functionally related analogue thereof. The method comprises inhibiting in plants a calcium dependant protein kinase or the production thereof and selecting the thus inhibited plants which are resistant and/or tolerant to the said agrochemical. In a further embodiment the method additionally comprises providing said plants with an amount of an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole. Also provided are polynucleotides which can be used in accordance with the methods of the invention.

HERBICIDE RESISTANT PLANTS AND METHODS FOR THE PRODUCTION THEREOF

The present invention relates *inter alia*, to herbicide resistant plants and methods for the production thereof. In particular, the invention relates to polynucleotide sequences and their use in the production of plants with an increased resistance and/or tolerance to a herbicide which comprises paraquat, diquat or a functionally related analogue thereof.

With the increasing world animal population there are now far greater demands on the land used to provide agriculturally related products. Farmers are faced with having to produce larger quantities of high quality crops in what are often unfavourable conditions. There are various factors which have an effect on the quality of the crop of interest such as poor soil quality; poor growing conditions; pests; and weeds growing in the same field. These weeds are problematic as they not only compete with the crop of interest for the nutrients contained within the soil but they can also act as host to various pests which are deleterious to the crop of interest. One option open to the professional agriculturist is to use herbicides to control the growth weeds.

Herbicides are usually classified by their action in the field and basically fall into two broad categories, *viz.*, non selective herbicides which are effective against a wide range of crops and selective herbicides which are only effective against certain crop groups. They are then sub divided into pre-emergence which are applied before the weeds and crop plants are established or post-emergence, being after the weeds (and often the crop plants) have become established. Some post emergence selective herbicides (which are not effective against the crop plant) can be applied to the entire crop/weed canopy. This is often described as "over-the-top application".

Paraquat and diquat are examples of non-selective post emergence contact herbicides both of which are inactivated in the soil. These herbicides will rapidly destroy a wide range of plants upon contact without leaving herbicidal residues in the soil which would otherwise be lethal to crops planted subsequently, making paraquat/diquat an ideal choice in removing weeds from fields prior to the planting/sowing of the crops of interest. These herbicides can not however, be used in an "over-the-top application" due to their non-selective nature. This leaves the farmer with a limited period within the growing season when paraquat/diquat can be applied.

The application of recombinant technology in the field of agriculture has huge potential for numerous benefits including cost and labour savings as well as improving production of high quality crops.

An example of the application of recombinant technology in this area is the production of crop plants which are resistant to herbicides thereby allowing the farmer to apply herbicides to fields comprising crop plants and weeds using "over-the-top application" throughout the growing season, the result being that the weeds are killed leaving the resistant crop plants in the field. This technology has been applied in amongst other things, the production of ROUNDUP READY™ Soya which is resistant to herbicides, having as a mode of action the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, such as those agrochemicals containing glyphosate.

Furthermore, International patent application PCT publication number WO99/32630 relates to plants which are resistant and/or tolerant to agrochemicals comprising paraquat, diquat or a functionally related analogue thereof through the production in plants of proteins which are capable of binding said paraquat, diquat or a functionally related analogue thereof.

Plants which are substantially "tolerant" to a herbicide when they are subjected to it provide a dose/response curve which is shifted to the right when compared with that provided by similarly subjected non tolerant like plants. Such dose/response curves have "dose" plotted on the x-axis and "percentage kill", "herbicidal effect" etc. plotted on the y-axis. Tolerant plants will typically require at least twice as much herbicide as non tolerant like plants in order to produce a given herbicidal effect. Plants which are substantially "resistant" to the herbicide exhibit few, if any, necrotic, lytic, chlorotic or other lesions when subjected to the herbicide at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Hereinafter the words (i) "tolerant" and (ii) "resistant" when used individually mean "tolerant and/or resistant".

The present invention seeks to provide *inter alia*, a method for the production of crop plants which are resistant and/or tolerant to an agrochemical comprising paraquat and/or diquat or a functionally related analogue thereof when applied at a concentration which is phytotoxic to non resistant/tolerant plants. The crops resulting from this method may be used as part of a weed control system in a way which would not be applicable to non-resistant like crop plants.

According to the present invention there is provided a method of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a functionally related analogue thereof characterised in that the method comprises inhibiting in plants a calcium dependant protein kinase (CDPK) or the production thereof and selecting the thus inhibited plants which are resistant and/or tolerant to the said agrochemical. The inhibition may be achieved, for example, via a chemical approach through the application of chemical CDPK inhibitors to the plant, via a transgenic approach through the transformation of plants with elements capable of providing for inhibition of the CDPK or alternatively through a combination of these techniques. The resistant and/or tolerant plants may be identified by their ability to survive when the agrochemical is applied to them at a concentration which is phytotoxic to control-like plants. A "control-like plant" is one which has been grown under substantially the same conditions as the inventive plant and is usually alike to the inventive plant in terms of its age, species etc., but it differs from the inventive plant in so far as its calcium dependant protein kinase is not inhibited, or is inhibited to a lesser extent, than that of the inventive plant. The control-like plant may be an untreated or untransformed plant or a plant which has undergone the transformation and regeneration process (a so-called transformation control), but without being provided with the elements required to inhibit the plants CDPK.

When making a comparison of phytotoxicity in the field, it will be apparent that it is only necessary to identify the concentration of agrochemical required to be phytotoxic to the weeds in the field without damaging the crop of interest.

The present invention further provides a method as described above comprising: (a) inserting into a plant cell a gene silencing vector adapted to inhibit production of a calcium dependant protein kinase; and (b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical. The plant cell may be any cell which is capable of regenerating into a plant. In a further embodiment of the present invention the method comprises: (a) inserting into a plant cell a polynucleotide comprising at least forty contiguous nucleotides of a sequence selected from the group depicted as SEQ ID No. 1 to 24 or at least forty contiguous nucleotides of a polynucleotide sequence which is the complement of one which binds to any one of the sequences depicted as SEQ ID Nos. 1 to 24 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed

by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and (b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical. In a further embodiment of the present invention said method uses the whole or a substantial part of the polynucleotide or polynucleotide sequence described above. In a still further embodiment of the present invention said polynucleotide comprises a sequence selected from the group consisting of SEQ ID No. 1; 2; 3; 4; 5; 6 or 7 or a polynucleotide sequence which binds to any one of the sequences selected from the group consisting of SEQ ID No. 1; 2; 3; 4; 5; 6 or 7 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase. The methods for the identification of further polynucleotide sequences are well known to the skilled man. Hybridisation conditions, such as those described above may be used or alternatively the polynucleotide sequence may be identified using the following hybridisation conditions: hybridisation step at a temperature of about 65°C in a solution containing 6 X SSC, 0.01% SDS and 0.25% skimmed milk powder, followed by rinsing step at the same temperature in a solution containing 0.2 X SSC and 0.1% SDS.

The calcium dependant protein kinase to be inhibited in accordance with the present invention may consist of a single polypeptide chain with a catalytic kinase domain within the amino terminus and a calcium binding domain within the carboxyl terminus. More specifically, the calcium dependant protein kinase to be inhibited may comprise a polynucleotide sequence comprising a region encoding the following amino acid sequence: His_1 Arg_2 Asp_3 Xaa_4 Lys_5 Pro_6 Glu_7 Asn_8 Xaa_9 Xaa_{10} - (SEQ ID No. 34) wherein the Xaa at position number 4 may be Leucine or Methionine; the Xaa at position number 9 may be Phenylalanine or Leucine; the Xaa at position number 10 may be Leucine or Threonine. In a further embodiment of the present invention the CDPK may be identified via the presence of a polynucleotide sequence selected from the group depicted as SEQ ID No. 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20; 21; 22; 23 and 24 or any degenerate sequence

thereof within the plant. Once the protein encoding region for the CDPK to be inhibited has been identified, it will be possible for the skilled man to create silencing vectors based on either that sequence or on other aspects of the gene which provide for the CDPK such as the CDPK promoter region, 5' untranslated region, intron sequences and the 3' untranslated region.

The present invention still further provides a method as described above comprising: (a) inserting into a plant cell a polynucleotide fusion which comprises a fusion between at least two polynucleotide sequences as described above wherein said polynucleotide fusion encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and (b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical. In a further embodiment of the present invention the polynucleotide fusion comprises the sequence depicted as SEQ ID No. 25 or 26. The polynucleotide fusion comprises a fusion (of at least two polynucleotides) between the 3' end and 5' end of the separate polynucleotides such that the sequences are preferably arrange in tandem. When the crop of interest contains a plurality of CDPK functions the polynucleotide fusion may be used to inhibit the CDPK activity of both genes. The polynucleotide fusion may also be used to provide a silencing vector with enhanced efficacy for CDPK inhibition. Alternatively a plurality of silencing vectors may be used to inhibit the CDPK activity or activities.

The present invention still further provides a method as described above which additionally comprises providing said plants with an amount of an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and selecting the plants which are resistant and/or tolerant to the said agrochemical. Such a method may comprise: (a) inserting into a plant cell a gene silencing vector adapted to inhibit production of the CDPK in the plant such as a first polynucleotide comprising at least forty nucleotides of the sequence selected from group depicted as SEQ ID No. 1 to 24, or at least forty nucleotides of a first polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 24 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said first polynucleotide sequence encodes either an RNA or a protein which is

inhibitory to production or function of the plants calcium dependant protein kinase; and (b) inserting into the plant cell a second polynucleotide comprising the sequence depicted as SEQ ID No. 35 or a second polynucleotide sequence which is the complement of one which binds to SEQ ID No. 35 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said second polynucleotide sequence encodes an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole; and (c) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical. Alternatively the method may comprise inserting into a plant cell a polynucleotide which comprises a first region which encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and a second region which provides for the production of an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

The present invention still further provides a method as described above comprising: crossing a first plant in which the calcium dependant protein kinase production is substantially inhibited with a second plant which is capable of producing an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and selecting the resultant plant which is resistant and/or tolerant to the said agrochemical

The present invention still further provides a method as described above wherein said polynucleotide, polynucleotide sequence, polynucleotide fusion, first polynucleotide, first polynucleotide sequence or first region are in antisense orientation. Thus, inhibition of the calcium dependant protein kinase may be achieved *inter alia*, by antisense inhibition or sense co-suppression. Antisense inhibition techniques are well developed and used routinely by persons skilled in the art. Inhibition is effected in the plant through the production of an antisense mRNA which is complementary to and capable of hybridising with the sense mRNA produced by the endogenous gene to be silenced. Sense co-suppression techniques are also well developed and used routinely by persons skilled in the art. Sense co-

suppression is effected through the insertion of the whole or part of a polynucleotide sequence which is preferably identical but may be substantially similar to the endogenous sequence encoding the target protein to be suppressed. The sense co-suppression vector may also comprise the whole or a part of a sequence which is preferably identical or substantially similar to the 5' untranslated region, the 3' transcription termination region or intron sequences of the endogenous gene encoding the target protein to be suppressed. In addition to this it has also been shown that insertion of regions which are substantially similar or identical to the promoter regions of the said endogenous gene will sometimes result in inhibition of production of the target protein.

The person skilled in the art will also appreciate that there are further ways of inhibiting or otherwise disrupting the function of a gene including, for example, modification of the promoter region of the endogenous gene, such as by deletion or mutation, resulting in an inability to bind RNA polymerase or otherwise inhibiting the endogenous gene by techniques such as specific DNA binding domain targeted methylation. In this case DNA methylases are targeted to specific binding domains within the gene to be suppressed thereby interrupting the endogenous target protein expression. Other techniques include excision of endogenous gene or interruption of expression via homologous recombination.

Another method uses the so called chimeroplasty technique of *in situ* mutagenesis of an endogenous gene. As with all of the inhibition methods described above, the technique *per se* is not germane to the present invention but, briefly, it involves the introduction into plant material of mixed ribo-deoxyribonucleic acids which comprise a region (typically less that 100 nucleotides in length) which is complementary to a target sequence in the endogenous gene, with the *proviso* that within the region of complementarily there is a "mismatch" which becomes represented in the endogenous gene *via* the action of DNA repair and replication enzymes. The mismatch typically occurs within a region of the gene encoding the active site of an enzyme, the activity of which is consequentially abolished or at least severely curtailed.

In summary then, any gene suppression technique can be applied in the method of the present invention. Additionally the person skilled in the art is also free to use techniques available within the art to enhance the efficacy of suppression of the desired gene. One such method involves the use of an inverted repeat sequence and is described in the International patent application, PCT publication number WO98/53083.

The present invention still further provides a method as described above wherein said polynucleotide, polynucleotide sequence, polynucleotide fusion, first polynucleotide, first polynucleotide sequence, second polynucleotide or second polynucleotide sequence is bounded by a plant operable promoter and a transcription termination sequence which is functional in plants. Such promoters and terminators, which are *per se* not germane to the invention, are well known to the skilled person and include, for example constitutive promoters such as the CaMV35S; FMV35S; NOS; OCS; Patatin and E9 (derived from the small subunit of RUBISCO). Inducibly, developmentally regulated and/or tissue specific promoters may also be used such as the oleosin; alcA/alcR switch; GST switch; RMS switch and ribulose bisphosphate carboxylase-oxygenase small sub-unit promoters. Terminators which can be used in the present invention include; NOS; proteinase inhibitor II and the terminator of a gene of alpha-tubulin (EP-A 652,286).

The present invention still further provides plants and plant progenitor material obtained according to a method as described above. In a further embodiment of the present invention said plants and plant progenitor material is selected from the group consisting of melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus, cereal, nut plants or other horticultural crops.

The present invention still further provides a method of providing a plant or plant part with a further desired agronomic trait comprising: (a) inserting into the genome of a plant or plant progenitor material as described above a polynucleotide which provides for the desired agronomic trait; and (b) regenerating plants or plant parts from said material; and (c) selecting the plants or plant parts having said further desired agronomic trait; or crossing a first plant which is resistant and/or tolerant to an agrochemical in accordance with the present invention with a second plant which provides for said further desired agronomic trait and selecting the resultant plant which is still resistant to said agrochemical and is capable of producing the further agronomic trait. In a further embodiment of the present invention the further desired agronomic trait is selected from the group consisting of: further herbicide resistance; insect resistance; nematode resistance; stress tolerance; altered yield; altered nutritional value or any other desirable agronomic trait.

The present invention still further provides plants or plant parts or plant progenitor material obtained according to the method of the preceding paragraph. Plant progenitor material includes all parts of the plant which are capable of exhibiting the resistance to the agrochemical in accordance with the present invention.

According to a further aspect of the present invention there is provided a polynucleotide consisting of the sequence selected from the group depicted as SEQ ID No. 1 to 7, 25 and 26. The present invention further provides an isolated polynucleotide comprising the sequence depicted as SEQ ID No. 8 to 24 in antisense orientation.

The present invention still further provides a DNA construct comprising in sequence a plant operable promoter region a polynucleotide as described in the preceding paragraph and a transcription termination region which is functional in plants. The DNA construct according to the invention may further comprises a polynucleotide encoding a selectable marker. The selectable marker may confer resistance to an antibiotic such as kanamycin; hygromycin or gentamycin. Further suitable selectable markers include genes which confer resistance to herbicides such as glyphosate based herbicides or resistance to toxins such as eutypine. Other forms of selection are also available such as hormone based selection systems such as the Multi Auto Transformation (MAT) system of Hiroyrasu Ebinuma *et al.* 1997. PNAS Vol. 94 pp2117-2121; visual selection systems which use the known green fluorescence protein, β glucoronidase and any other selection system such as mannose isomerase, xylose isomerase and 2-deoxyglucose (2-DOG).

The polynucleotide/DNA construct may be inserted into the cells in accordance with the methods of the present invention by plant transformation techniques which are well known to the person skilled in the art. Such techniques include but are not limited to particle mediated biolistic transformation, *Agrobacterium*-mediated transformation, protoplast transformation (optionally in the presence of polyethylene glycols); sonication of plant tissues, cells or protoplasts in a medium comprising the polynucleotide or vector; micro-insertion of the polynucleotide or vector into totipotent plant material (optionally employing the known silicon carbide "whiskers" technique), electroporation and the like.

In a further aspect of the present invention there is provided the use of a DNA construct as described above or at least forty nucleotides of a polynucleotide selected from the group depicted as SEQ ID No. 1 to 26 or at least forty nucleotides of a polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 26 at

a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase in a method of producing plants which are resistant and/or tolerant to an agrochemical comprising paraquat, diquat or a functionally related analogue thereof. In a further embodiment of the present invention there is provided the use of a polynucleotide selected from the group depicted as SEQ ID No. 1 to 26 or a polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 26 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase in a method of producing plants which are resistant and/or tolerant to an agrochemical comprising paraquat, diquat or a functionally related analogue thereof. In a still further embodiment of the present invention the said polynucleotide or polynucleotide sequence used is in antisense orientation.

The present invention also extends to the use of further polynucleotides which are inhibitory to the production of plant calcium dependant protein kinases. Such polynucleotides may be identified from other organisms through the use of known molecular biology techniques including nucleic acid library screening using the sequence information described herein to construct oligonucleotide probes. The person skilled in the art is well versed in such methods for the production and screening of nucleic acid libraries (such as gDNA/cDNA) and the necessary techniques for the subsequent identification, isolation and sequence determination of further polynucleotides which can be used in gene silencing programmes. The skilled man will also appreciate that other methods exist for the identification of further CDPK sequences through the use of PCR strategies again based on oligonucleotide primers using the sequence information provided herein. Such further CDPK sequences when identified, can also be used to produce gene silencing vectors to be used in accordance with the methods of the present invention.

The present invention still further provides a method of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a

functionally related analogue thereof comprising (a) inserting into a plant cell a polynucleotide which is at least forty contiguous nucleotides and wherein part of said polynucleotide encodes the motif depicted as SEQ ID No. 34 and wherein said polynucleotide encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and (b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

In a further aspect of the present invention there is provided a method of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a functionally related analogue thereof characterised in that the method comprises providing the plants with an amount of an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and selecting those resultant plants which are resistant and/or tolerant to the said agrochemical. In a further embodiment of the present invention said method comprises: (a) inserting into a plant cell a polynucleotide encoding the protein sequence depicted as SEQ ID No. 36 or an active protein variant or polynucleotide sequence which is the complement of one which binds to a polynucleotide which encodes the protein depicted as SEQ ID No. 36 or said variant at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole; and

(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical. In a further embodiment of the present invention said polynucleotide comprises the sequence depicted as SEQ ID No. 35. A protein variant is an active protein which may be achieved through conservative substitutions within the amino acid sequence which substitutions do not significantly adversely affect the agrochemical binding and vacuolar transport activity of the protein. In particular substitutions may be made between the following amino acid groups viz.:

(i) Alanine and Glycine;

(ii) Serine and Threonine;

(ii) Glutamic acid and Aspartic acid;

(iii) Arginine and Lysine;

(iv) Asparagine and Glutamine;

(v) Isoleucine and Leucine,

(vi) Valine and Methionine;

(vii) Phenylalanine and Tryptophan.

In general, more conservative than non-conservative substitutions will be possible without destroying the vacuolar transporter properties of the proteins. Suitable variant proteins in accordance with the present invention may be determined and tested using routine methods which are well known to the person skilled in the art. Such variant proteins may also be synthesised chemically using standard techniques. The present invention also extends to the use of further intracellular vacuolar transporters which may be identified from other organisms through the use of known molecular biology techniques involving homology screening of further organisms' nucleic acid libraries (such as gDNA/cDNA) and the subsequent identification and isolation of the intracellular vacuolar transporter sequences. The person skilled in the art will appreciate that alternative methods exist for the identification and characterisation of further vacuolar transporters from other sources such as plants. Such methods include PCR strategies based on oligonucleotide primers using, or deduced from the sequence information provided herein or from the further sequences obtained using the screening techniques described above.

The polynucleotide encoding said vacuolar transporter may be bounded by a plant operable promoter and terminator such as those described above. Furthermore, it is possible to use, in association with the promoter sequence, other regulation sequences which are situated between the promoter and the sequence encoding the vacuolar transporter protein used in the methods of the present invention, such as transcriptional or translational enhancers, for example, tobacco etch virus (TEV) translation activator described in International Patent application, PCT publication number WO87/07644. The polynucleotide encoding the vacuolar transporter protein used in the methods of the present invention may also be codon-optimised, or otherwise altered to enhance for example, transcription once it is incorporated into plant material. An example of preferred codon usage from cotton and maize plants is set out in Table 1 below.

Table 1

Amino Acid	Cotton preference	Maize preference
Alanine	GCT	GCC
Arginine	AGG	AGG
Asparagine	AAC	ACC
Aspartic Acid	GAT	GAC
Cysteine	TGC	TGC
Glutamine	CAA	CAG
Glutamic Acid	GAG	GAG
Glycine	GGT	GGC
Histidine	CAT	CAC
Isoleucine	ATT	ATC
Leucine	CTT	CTG
Lysine	AAG	AAG
Methionine	ATG	ATG
Phenylalanine	TTC	TTC
Proline	CCT	CCG
Serine	TCT	AGC
Threonine	ACT	ACC
Tryptophan	TGG	TGG
Tyrosine	TAC	TAC
Valine	GTT	GTG

Such codon optimisation may also be used to alter the predicted secondary structure of the RNA transcript produced in any transformed cell, or to destroy cryptic RNA instability elements present in the unaltered transcript, thereby increasing the stability and/or availability of the transcript in the transformed cell (Abler and Green. 1996. Plant Molecular Biology (32) pp63-78). The expression of the vacuolar transporter protein used in the methods of the present invention may also be enhanced through the inclusion of one or more intron sequences within the polynucleotide encoding said protein and/or combination. (Rose and

Beliakoff, 2000. Plant Physiology (122) pp.535-542). Examples of such sequences are the second intron of the *Solanum tuberosum* LS1 gene and the alcohol dehydrogenase 1 gene (*adh1*) intron of monocotyledonous plant species.

The present invention still further provides plants, plant parts and plant progenitor material obtained by the methods as described above.

The present invention still further provides the use of a vacuolar transporter in a method of producing plants which are resistant and/or tolerant to an agrochemical comprising paraquat, diquat or a functionally related analogue thereof.

The present invention still further provides a method of selectively controlling weeds in a field said field comprising crop plants and weeds said method comprising applying to said field an agriculturally acceptable formulation of an agrochemical comprising paraquat, diquat or a functionally related analogue thereof, characterised in that the said crop plants are the plants as mentioned above.

The invention will now be further illustrated by way of the following non-limiting examples and with reference to the sequence listing and figures of which:

SEQ ID No. 1 = *Arabidopsis sp.* calcium dependant protein kinase clone 23.1.

SEQ ID No. 2 = *Arabidopsis sp.* calcium dependant protein kinase clone 15.1.

SEQ ID No. 3 = PCR product of part of clone 15.1.

SEQ ID No. 4 = PCR product of part of clone 23.1.

SEQ ID No. 5 = *Arabidopsis sp.* calcium dependant protein kinase clone.

SEQ ID No. 6 = *Arabidopsis sp.* calcium dependant protein kinase clone.

SEQ ID No. 7 = *Arabidopsis sp.* calcium dependant protein kinase clone.

SEQ ID No. 8 = EMBL: Y18055 *Arachis hypogea.*

SEQ ID No. 9 = EMBL: DCPK431 *Daucus carota.*

SEQ ID No. 10 = EMBL: ZM28376 *Zea mays.*

SEQ ID No. 11 = EMBL: ZMCDPK1 *Zea mays.*

SEQ ID No 12 = EMBL: U69174 *Glycine max.*

SEQ ID No 13 = EMBL: GMCADPK *Glycine max.*

SEQ ID No 14 = EMBL: IBD707 *Ipomoea batatas.*

SEQ ID No 15 = EMBL: AF072908 *Nicotiana tabacum.*

SEQ ID No 16 = EMBL: X81393 *Oryza sativa.*

SEQ ID No 17 = EMBL: OSCDPK2 *Oryza sp.*

SEQ ID No 18 = EMBL: AF030879 *Solanum tuberosum.*

SEQ ID No 19 = EMBL: AF035944 *Fragaria x ananassa.*

SEQ ID No 20 = EMBL: AB017517 *Marchantia polymorpha.*

SEQ ID No 21 = EMBL: AB017516 *Marchantia polymorpha.*

SEQ ID No 22 = EMBL: U90262 *Cucurbita pepo.*

SEQ ID No 23 = EMBL: AF090835 *Mesembryanthemum crystallinum.*

SEQ ID No 24 = EMBL: U08140 *Vigna radiata.*

SEQ ID No. 25 and 26 = PCR product of fusion between part of clone 23.1 and 15.1.

SEQ ID No. 27 to 33 = PCR primers.

SEQ ID No. 34 = CDPK signature domain.

SEQ ID No. 35 = EMBL: Y13138 *S. cerevisiae* YLL028w/TPO1 vacuolar transporter.

SEQ ID No. 36 = Protein sequence encoded by SEQ ID No. 35.

FIGURE 1 is the construct pBCDPK19 containing the CDPK19 PCR product in antisense orientation.

FIGURE 2 is the construct pBCDPK23 containing the CDPK23 PCR product in antisense orientation.

FIGURE 3 is the construct pBCDPK1923 containing the CDPK19 and 23 PCR products, both of which are in antisense orientation.

Example 1

Production of plants resistant/tolerant to paraquat through antisense inhibition of the plant calcium dependant protein kinase (CDPK).

1.1 Isolation and preparation of polynucleotide sequence for production of antisense constructs

DNA sequences used for the production of the antisense constructs pBCDPK19, pBCDPK23 and pBCDPK1923 (Figures 1, 2 and 3) are generated by PCR from the plasmid clones depicted as SEQ ID No. 1 and SEQ ID No. 2.

The following oligonucleotide primers are synthesised by Gibco BRL™ according to standard methods:

19FBam	ACCCGGATCCAGGAGCTTAAATTTGGACTTCA
19RXho	TTTCTCGAGGTCCACAACATGAAAATAAAGAGTCAG
19RXho	5' - AAACACTCGAGACCCTGATCTACAATTATTGAGG -3'
23FBam	ACTCGGATCCAGGGTTATTGCCGAATTTTTATCTACGG
23RXho	TTCCTCGAGATCCCAAGTTTTACCTTCTCTTGGT
23FSac	ACTCGAGCTCAGGGTTATTGCCGAATTTTTATCTACGG
23RBam	TTCGGATCCATCCCAAGTTTTACCTTCTCTTGGT

Primers 19FBam and 19RXho are used to amplify a 593 bp region from the 3' end of clone 15.1, resulting in the incorporation of *Bam*H1 and *Xho*I restriction sites onto the 5' and 3' termini respectively of the PCR product (SEQ ID No.3).

Primers 23FBam and 23RXho are used to amplify a 676 bp region from the 3' end of clone 23.1, resulting in the incorporation of *Bam*H1 and *Xho*I restriction sites onto the 5' and 3' termini respectively of the PCR product (SEQ ID No. 4).

Primers 23FSac and 23RBam are used to amplify the same 676 bp region from the 3' end of clone 23.1 resulting in the incorporation of *Sac*I and *Bam*H1 restriction sites onto the 5' and 3' termini respectively of the PCR product.

PCR products are analysed by gel electrophoresis to ensure specificity. Subsequently products were purified using the Qiagen™ PCR clean up kit according to manufacturers instructions prior to restriction endonuclease digestion. Restriction endonuclease digests are performed using standard procedures.

1.2. Production of antisense construct pBCDPK19

The product is generated by digestion of the purified PCR product of clone 15.1 with *Bam*HI and *Xho*I restriction endonucleases, followed by ligation into the respective sites of the plasmid pMJB1. The resulting intermediate plasmid pMJB19 contains the CDPK19 PCR

product in the antisense orientation, flanked upstream by a double CaMV 35S promoter and downstream by the NOS terminator. This cassette is isolated by digestion of pMJB19 with *Hind*III and *Eco*RI, and agarose gel electrophoresis. The DNA is visualised under UV light and the band of predicted size is removed. DNA is then purified from the gel using the Qiagen™ gel purification kit according to known techniques. The cassette is then inserted into the *Hind*III, *Eco*RI restriction sites of the binary vector pBIN19 and subsequently transformed into competent *E.coli* cells according known techniques. Following cloning into the binary vector, pBIN19 junction regions are sequenced to confirm correct re-ligation.

1.3. Production of antisense construct pBCDPK23

The production of this construct is carried out essentially as described in part 1.2 above with the exception that the initial PCR product used is generated using the 23.1 clone as DNA template and 23FBam and 23RXho oligonucleotide primers.

1.4. Production of antisense construct pBCDPK1923

The PCR product of clone 23.1B generated using the primers 23FSac and 23RBam is digested with *Sac*I and *Bam*HI restriction endonucleases and ligated into the respective sites of pMJB19. The resultant plasmid pMJB1923 contains the specified 3' regions of both CDPK19 and CDPK 23 (SEQ ID No. 25) in antisense orientation flanked by the double CaMV 35S promoter and the NOS terminator as previously described in part 1.2 above. The promoter, antisense DNA, and terminator are isolated as a cassette using *Hind*III and *Eco*RI restriction sites and cloned into the binary vector as previously described in part 1.2 above.

1.5. Sequencing

In order to confirm the correct antisense sequence is cloned, the generated plasmids pMJB19,pMJB23, pMJB1923 are then sequenced using the known methods for the machine used (ABI™ DNA sequencer). Oligonucleotides are generated allowing the region containing the antisense DNA to be sequenced in both forward and reverse orientations.

1.6. Plant transformation/regeneration

Constructs pBCDPK19, pBCDPK23 and pBCDPK1923 are transformed into *Agrobacterium* strain by electroporation according to known methods. Transformation into *Arabidopsis* is performed using standard infiltration methods such as those described in Clough & Bent 1998 and Bechtold & Pelletier, 1998. Transgenic material is selected by plating out onto MS media containing kanamycin. Selected lines are then grown to maturity and self-pollinated to bulk up seed quantity.

1.7.Analysis of Transgenic Plants.

PCR analysis of transformants

PCR analysis is performed on leaf material from primary transformants to ensure the presence of the antisense construct. Oligonucleotide primers are synthesised by Gibco BRL™ to regions within the T-DNA borders. Leaf material is prepared according to Klimyuk *et al.* (1993) and PCR is carried out using standard procedures.

RNA analysis

The effect of the antisense constructs on levels of endogenous CPDK mRNA is examined in selected transgenic lines by Northern blot hybridisation. RNA is extracted from leaf tissue using TriZOL (Gibco BRL™) procedure according to the known techniques. Denaturing RNA gels and Northern blots are prepared according to known methods (Sambrook *et al.*) and filters hybridised using probes prepared from DNA fragments present within SEQ ID No.1 and SEQ ID No. 2.

Protein Analysis

Oligonucleotides are synthesised and used to amplify by PCR the sequence encoding either CDPK19 or CPDK 23 proteins regions and fragments thereof. The PCR products are cloned into the over-expression vector pQE-60 (Qiagen™), transformed into *E.coli* TOP 10 cells for expression of the protein. The over-expressed protein was purified using the 6 x His Tag with Ni-NTA resin (Qiagen™) and used as antigen for production of antiCDPK antibodies. Crude soluble protein extracts are prepared from leaf tissue of transgenic plants. SDS-PAGE is performed on Tris-Glycine gels (Novex) and proteins blotted onto PVDF membrane using

known methods. Levels of endogenous CDPK19 and 23 proteins are measured by Western blot analysis against known standards using raised antiCDPK antibodies and ECL Kit (Amersham™) using known methods.

Paraquat tolerance trials

Plants are transferred to 5 inch pots containing John Innes potting compost No. 3 and evaluated by post emergence foliar application of differing concentrations of liquid paraquat. Visual assessment of phytotoxicity is carried out after 5 and 17 days after treatment. Transgenic lines with inhibited endogenous CDPK show resistance to concentrations of paraquat which are phytotoxic to control like plants.

Analysis of chlorophyll content of leaf discs

Paraquat is also added to molten MS agar to a final concentration of 2, 5, 10 and 20 μM, prior to pouring. Leaf discs are isolated from transgenic plants and placed on the surface of the solidified media and sealed in petri dishes. The dishes are then placed under illumination for 48 hours, and plants assessed for visual damage. Measurement of chlorophyll content is performed as a quantitative assessment of herbicide damage. Transgenic lines with inhibited endogenous CDPK show a higher chlorophyll content than the control-like plants.

Production of homozygous plant lines

Single copy transgenic plant lines are identified from selected lines by Southern hybridisation according to the methods described in Sambrook *et al.* Isolated genomic DNA is digested with a suitable restriction enzyme, e.g. *Eco*RI, separated on an agarose gel, and transferred to nylon filters. DNA is then hybridised with a radiolabelled probe prepared from the NOS terminator DNA fragment. Hybridisation is carried out at 65°C for 16 hours and filters washed at high stringency.

After identification of single insert lines, plants are self pollinated and the resulting seed are analysed for segregation by germination on MS media containing kanamycin. Further confirmation of homozygous lines is performed by back-crossing selected lines to wildtype

Arabidopsis and analysing seed segregation by germination on MS media containing kanamycin.

References

Bechtold N & Pelletier G (1998) *In planta* Agrobacterium-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol 82: 259-266.

Clough & Bent (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743

Klimyuk VI, Carroll BJ, Thomas CM, Jones JDG. (1993) Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J. 3: 493-494

Sambrook J (1989) Molecular Cloning: A laboratory Manual. 2nd Edition. CSH Laboratory Press.

CLAIMS

1. A method of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a functionally related analogue thereof characterised in that the method comprises inhibiting in plants a calcium dependant protein kinase or the production thereof and selecting the thus inhibited plants which are resistant and/or tolerant to the said agrochemical.

2. A method according to claim 1 comprising:
(a) inserting into a plant cell a gene silencing vector adapted to inhibit production of a calcium dependant protein kinase; and
(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

3. A method according to claim 1 or claim 2 comprising:
(a) inserting into a plant cell a polynucleotide comprising at least forty contiguous nucleotides of a sequence selected from the group depicted as SEQ ID No. 1 to 24 or at least forty contiguous nucleotides of a polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 24 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and
(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

4. A method according to claim 3 wherein said polynucleotide comprises a sequence selected from the group consisting of SEQ ID No. 1 to 7.

5. A method according to claim 1 comprising:
(a) inserting into a plant cell a polynucleotide fusion which comprises a fusion between at least two polynucleotide sequences according to claim 3 wherein said polynucleotide fusion encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and
(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

6. A method according to claim 5 wherein the polynucleotide fusion comprises the sequence depicted as SEQ ID No. 25 or 26.

7. A method according to claim 1 which additionally comprises providing said plants with an amount of an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and selecting the plants which are resistant and/or tolerant to the said agrochemical.

8. A method according to claim 7 comprising:
(a) inserting into plant cell a first polynucleotide comprising at least forty contiguous nucleotides of the sequence selected from group depicted as SEQ ID No. 1 to 24 or at least forty contiguous nucleotides of a first polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 24 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said first polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and
(b) inserting into the plant cell a second polynucleotide comprising the sequence depicted as SEQ ID No. 35 or a second polynucleotide sequence which is the complement of one which binds to SEQ ID No. 35 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing

0.1% SDS wherein said second polynucleotide sequence encodes an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole; and

(c) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

9. A method according to claim 7 comprising:

crossing a first plant in which the calcium dependant protein kinase production is substantially inhibited with a second plant which is capable of producing an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole and selecting the resultant plant which is resistant and/or tolerant to the said agrochemical.

10. A method according to any one of claims 3 to 6 or 8 wherein said polynucleotide, polynucleotide sequence, polynucleotide fusion, first polynucleotide or first polynucleotide sequence are in antisense orientation.

11. A method according to any one of claims 3 to 6, 8 to 10 wherein said polynucleotide, polynucleotide sequence, polynucleotide fusion, first polynucleotide, first polynucleotide sequence, second polynucleotide or second polynucleotide sequence is bounded by a plant operable promoter and a transcription termination sequence which is functional in plants.

12. Plants and plant progenitor material obtained according to a method of any one of claims 1 to 11.

13. Plants and plant progenitor material according to claim 12 selected from the group consisting of melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine,

poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus, cereal, nut plants or other horticultural crops.

14. A method of providing a plant or plant part with a further desired agronomic trait comprising:

(a) inserting into the genome of a plant or plant progenitor material according to claim 12 or 13 a polynucleotide which provides for the desired agronomic trait; and

(b) regenerating plants or plant parts from said material; and

(c) selecting the plants or plant parts having said further desired agronomic trait; or

crossing a first plant according to claim 12 or 13 with a second plant which provides for said further desired agronomic trait and selecting the resultant plant which is capable of producing the further agronomic trait.

15. A method according to claim 14 wherein the further desired agronomic trait is selected from the group consisting of: further herbicide resistance; insect resistance; nematode resistance; stress tolerance; altered yield; altered nutritional value or any other desirable agronomic trait.

16. Plants or plant parts obtained according to the method of claims 14 or 15.

17. A polynucleotide consisting of the sequence selected from the group depicted as SEQ ID No. 1 to 7, 25 and 26.

18. A polynucleotide according to claim 17 or an isolated polynucleotide comprising the sequence depicted as SEQ ID No. 8 to 24 arranged in antisense orientation.

19. A DNA construct comprising in sequence a plant operable promoter region a polynucleotide according to claim 17 or claim 18 and a transcription termination region which is functional in plants.

20. A DNA construct according to claim 19 which further comprises a polynucleotide encoding a selectable marker.

21. Use of a DNA construct according to claim 19 or claim 20 or at least forty nucleotides of a polynucleotide selected from the group depicted as SEQ ID No. 1 to 26 or at least forty nucleotides of a polynucleotide sequence which is the complement of one which binds to any one of SEQ ID Nos. 1 to 26 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase in a method of producing plants which are resistant and/or tolerant to an agrochemical comprising paraquat, diquat or a functionally related analogue thereof.

22. Use according to claim 21 wherein said polynucleotide or polynucleotide sequence is in antisense orientation.

23. A method according to claim 1 comprising:
(a) inserting into a plant cell a polynucleotide which is at least forty contiguous nucleotides and wherein part of said polynucleotide encodes the motif depicted as SEQ ID No. 34 and wherein said polynucleotide encodes either an RNA or a protein which is inhibitory to production or function of the plants calcium dependant protein kinase; and
(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

24. A method of providing plants which are resistant and/or tolerant to an agrochemical which comprises paraquat, diquat or a functionally related analogue thereof characterised in that the method comprises providing the plants with an amount of an intracellular vacuolar transporter which is capable of binding and transporting said

agrochemical to the plant intracellular vacuole and selecting those resultant plants which are resistant and/or tolerant to the said agrochemical.

25. A method according to claim 24 comprising:

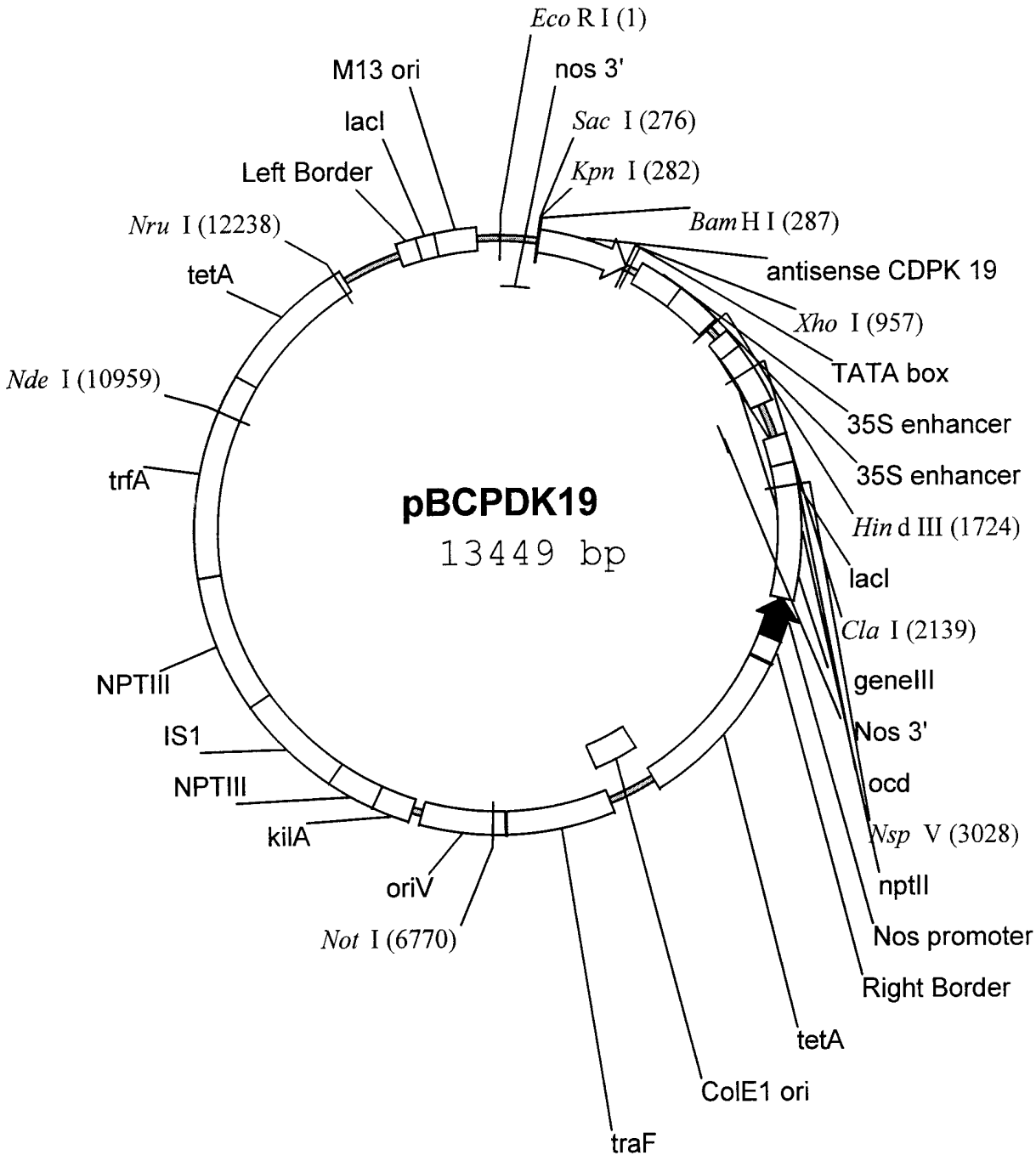
(a) inserting into a plant cell a polynucleotide encoding the protein depicted as SEQ ID No. 36 or a protein variant or polynucleotide sequence which is the complement of one which binds to a polynucleotide which encodes the protein depicted as SEQ ID No. 36 or said variant at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence encodes an intracellular vacuolar transporter which is capable of binding and transporting said agrochemical to the plant intracellular vacuole; and

(b) regenerating plants or plant parts therefrom and selecting from the population of regenerants those plants or plant parts which are resistant and/or tolerant to the said agrochemical.

26. A method according to claim 25 wherein said polynucleotide comprises the sequence depicted as SEQ ID No. 35.

27 A method of selectively controlling weeds in a field said field comprising crop plants and weeds said method comprising applying to said field an agriculturally acceptable formulation of an agrochemical comprising paraquat, diquat or a functionally related analogue thereof, characterised in that the said crop plants are the plants according to claim 12, 13 or 16.

Fig. 1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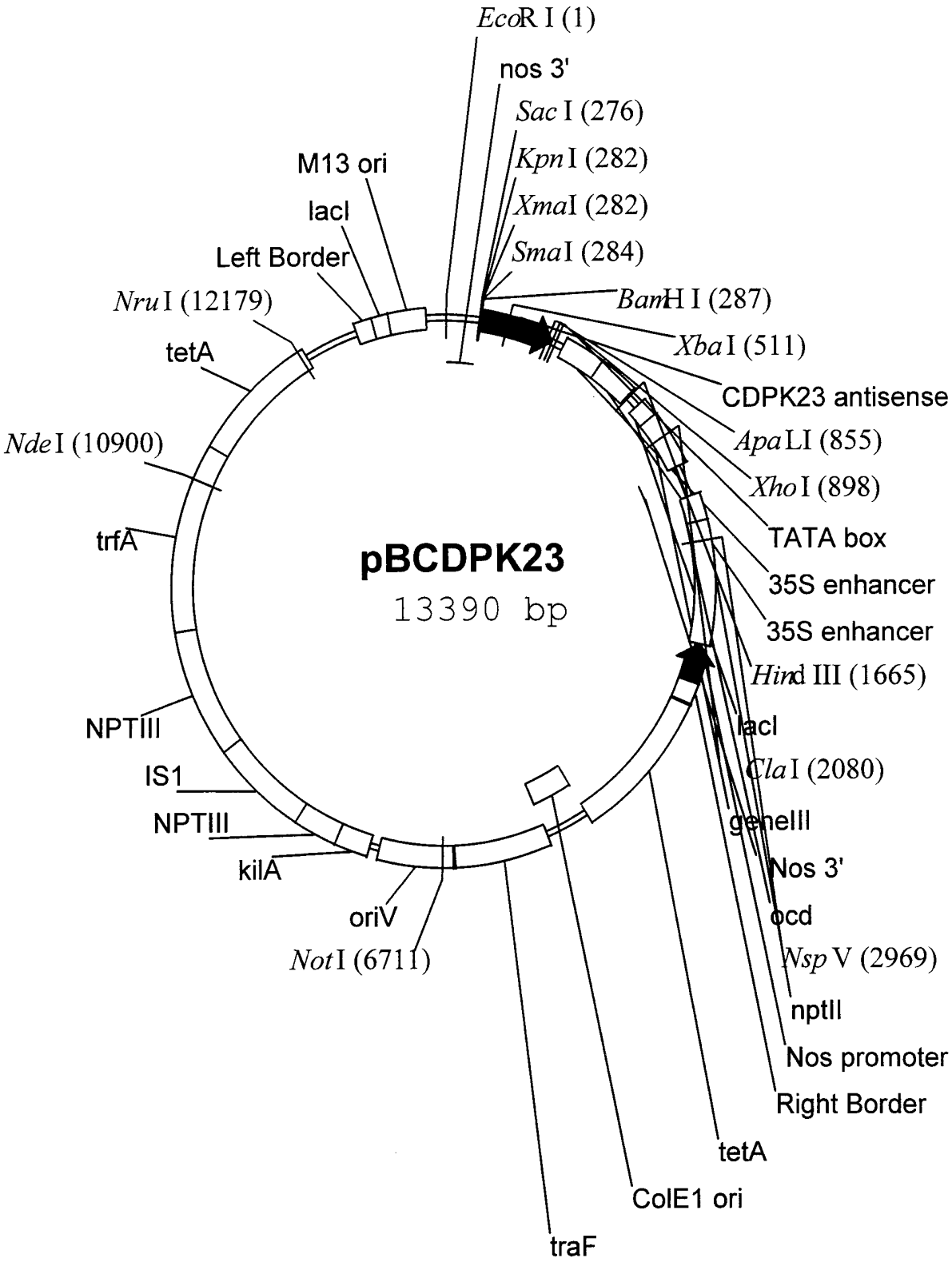
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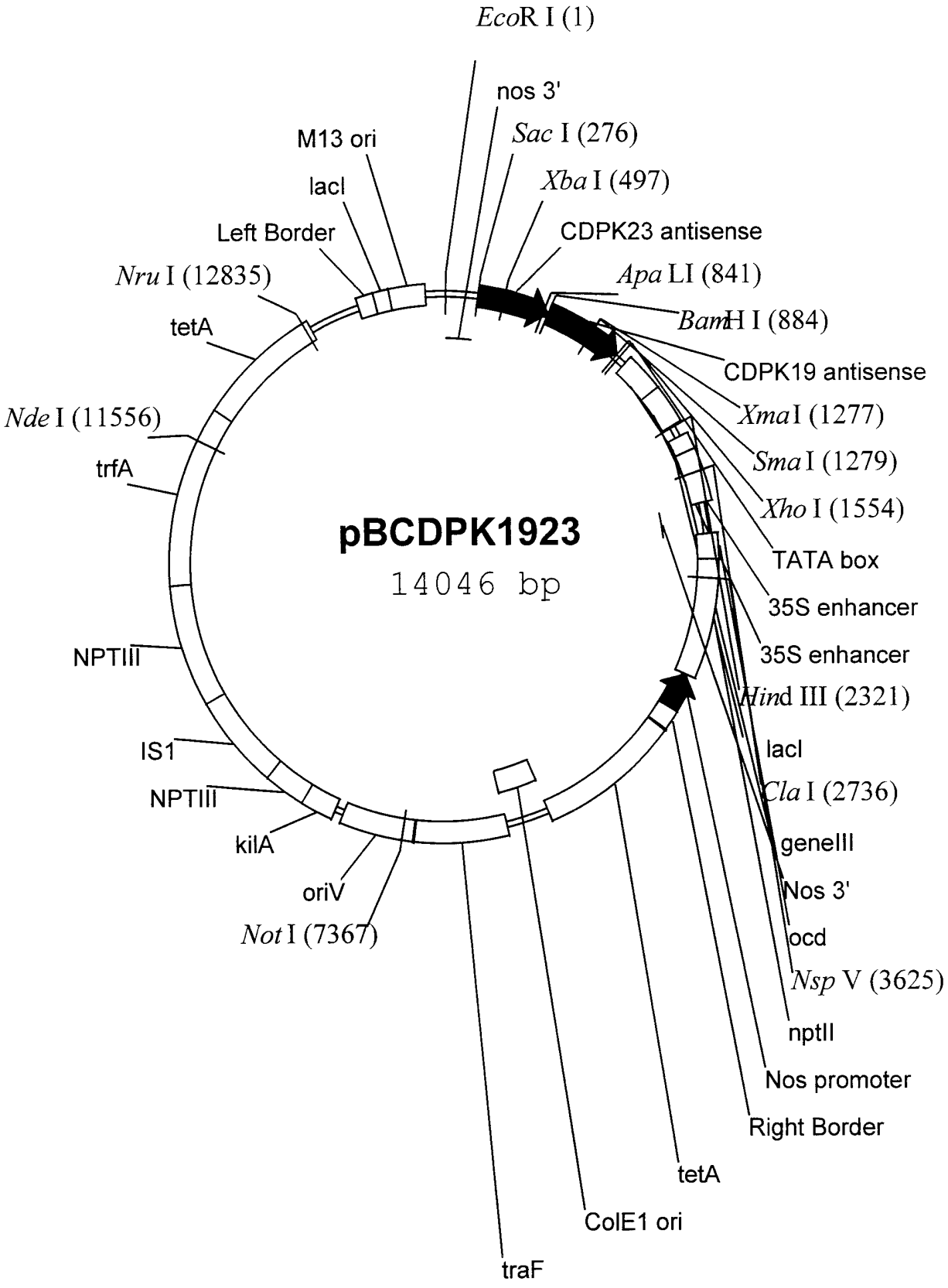
Fig. 3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<213> Glycine max

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<212> DNA
<213> Oryza sativa

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<212> DNA

<213> Oryza sativa

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<213> Marchantia polymorpha

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FUSION CDPK SILENCING VECTOR

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tctgtctctc acgccacgtt ctcgtgcacg tcaagtatta aaccaagaga aggtaaaact 660
tgggatggat ccaggagctt aaatttggac ttcataaact cggacagcag cagatacctg 720
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<210> 26
<211> 1288
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:POLYNUCLEOTIDE
FUSION CDPK SILENCING VECTOR

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ctcttggaga tgttgtgaag tcgtggatac taaaggaaaa ggaactctag actatggaga 240
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<210> 27
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 27
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<210> 28
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 28
tttctcgagg tccacaacat gaaaataaag agtcag 36

<210> 29
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 29
aaacactcga gaccctgatc tacaattatt gagg 34

<210> 30
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 30
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<210> 31
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 31

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<210> 32
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 32
actcgagctc agggttattg ccgaattttt atctacgg 38

<210> 33
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR PRIMER

<400> 33
ttcggatcca tcccaagttt taccttctct tggt 34

<210> 34
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:KINASE
SIGNATURE DOMAIN

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<222> (4)
<223> X is Leu or Met

<220>
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<223> X is Leu or Thr

<400> 34
His Arg Asp Xaa Lys Pro Glu Asn Xaa Xaa
1 5 10

<210> 35
<211> 3692
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<223> Description of Artificial Sequence

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aaacattacc ttcatttttt tacggccgga gaggggtagg tacacttaac cccgagcgct 180
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<210> 36
<211> 586
<212> PRT

<213> Saccharomyces cerevisiae

<220>
<223> Description of Artificial Sequence

<400> 36

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Met Ser Asp His Ser Pro Ile Ser Asn Lys Glu Asn His Leu Leu Pro
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Thr Gly Thr Thr Gly Val Glu Pro Val Asp Phe Thr Gly Glu Gly Ala
         35                  40                  45

Lys Tyr Thr Thr Ala Thr Glu Gly Asn Gly Gly Ala Asp Leu Ala Ile
     50                  55                  60

Gln Arg Thr Thr Thr Met Asn Ser Ala Ala Glu Ser Glu Val Asn Ile
 65                  70                  75                  80

Thr Arg Arg Leu Thr Lys Ile Leu Thr Gly Ser Val Asn Glu Pro Asp
                 85                  90                  95

Arg Val Glu Val Asp Tyr Thr Asn Cys Ala Pro Met Gly Gly Asp Arg
            100                 105                 110

Pro Tyr Pro Pro Ser Leu Pro Ser Arg Asp Leu Tyr Glu Val Thr Phe
        115                 120                 125

Asp Gly Pro Asn Asp Pro Leu His Pro Phe Asn Trp Pro Met Lys Lys
    130                 135                 140

Lys Val Leu Leu Cys Leu Val Leu Cys Leu Asp Ser Ile Ala Ile Ala
145                 150                 155                 160

Met Cys Ser Ser Ile Phe Ala Ser Ala Val Pro Gln Ile Cys Glu Ile
                165                 170                 175

Tyr His Val Ile Glu Val Val Ala Ile Leu Gly Ile Thr Leu Phe Val
            180                 185                 190

Leu Gly Phe Ala Ala Ser Pro Val Ile Tyr Ala Pro Leu Ser Glu Leu
        195                 200                 205

Tyr Gly Arg Lys Gly Val Leu Val Leu Ser Ala Phe Gly Phe Ala Leu
    210                 215                 220

Phe Gln Phe Ala Val Ala Thr Ala Glu Asn Leu Gln Thr Ile Phe Ile
225                 230                 235                 240

Cys Arg Phe Phe Gly Gly Phe Ile Gly Ala Ala Pro Met Ala Val Val
                245                 250                 255

Pro Ala Ala Phe Ala Asp Met Phe Asp Thr Asn Val Arg Gly Lys Ala
            260                 265                 270

Ile Ala Leu Phe Ser Leu Gly Val Phe Val Gly Pro Ile Leu Ser Pro
        275                 280                 285

Val Met Gly Ser Tyr Ile Ala Gln Arg Thr Thr Trp Arg Trp Leu Glu
    290                 295                 300

Tyr Val Val Gly Cys Phe Ala Ser Ala Val Phe Val Ala Ile Val Leu
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305 310 315 320

Phe Phe Glu Glu Thr His His Pro Thr Ile Leu Val Asn Lys Ala Lys
325 330 335

Gln Met Arg Lys Gln Ser Asn Asn Trp Gly Ile His Ala Ala His Glu
340 345 350

Asp Val Glu Leu Ser Ile Lys Asp Ile Val Gln Lys Thr Val Thr Arg
355 360 365

Pro Ile Ile Met Leu Phe Val Glu Pro Leu Leu Leu Phe Val Thr Ile
370 375 380

Tyr Asn Ser Phe Val Tyr Gly Ile Leu Tyr Leu Leu Leu Glu Ala Tyr
385 390 395 400

Pro Leu Val Phe Val Glu Gly Tyr Gly Phe Thr Glu Asn Gly Glu Leu
405 410 415

Pro Tyr Ile Ala Leu Ile Ile Gly Met Met Val Cys Ala Ala Phe Ile
420 425 430

Trp Tyr Met Asp Asn Asp Tyr Leu Lys Arg Cys Arg Ala Lys Gly Lys
435 440 445

Leu Val Pro Glu Ala Arg Leu Tyr Ala Met Val Ile Ala Gly Thr Val
450 455 460

Phe Pro Ile Gly Ile Leu Trp Phe Cys Trp Thr Gly Tyr Tyr Pro His
465 470 475 480

Lys Ile His Trp Met Val Pro Thr Val Gly Gly Ala Phe Ile Gly Phe
485 490 495

Gly Leu Met Gly Ile Phe Leu Pro Cys Leu Asn Tyr Ile Ile Glu Ser
500 505 510

Tyr Leu Leu Leu Ala Ala Ser Ala Val Ala Ala Asn Thr Phe Met Arg
515 520 525

Ser Ala Phe Gly Ala Cys Phe Pro Leu Phe Ala Gly Tyr Met Phe Arg
530 535 540

Gly Met Gly Ile Gly Trp Ala Gly Leu Leu Leu Gly Leu Phe Ala Ala
545 550 555 560

Ala Met Ile Pro Val Pro Leu Leu Phe Leu Lys Tyr Gly Glu Ser Ile
565 570 575

Arg Lys Lys Ser Lys Tyr Ala Tyr Ala Ala
580 585