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(54) Title: PHOSPHATE STARVATION-INDUCIBLE PROTEINS

(57) Abstract

This invention provides proteins, especially protein kinases, glucosidases, and phosphate transporters which are expressed under conditions of phosphate deprivation. Further provided are nucleic acids and nucleic acid constructs encoding these proteins, cells containing the nucleic acids described and transgenic photosynthetic organisms with altered phosphate-inducible enzyme activity.

PHOSPHATE STARVATION-INDUCIBLE PROTEINS

Background of the Invention

Phosphorus is one of the most important nutrients for plants. It is essential for their growth and is a structural component of nucleic acids, phospholipids, intermediary metabolites and numerous other biological molecules.

In plants, the only readily absorbed form of exogenous phosphorus is inorganic phosphate (P_i) (Bieleski, 1973). When the amount of available phosphate is low, plants are unable to grow vigorously and productively. When phosphate is absent, growth is halted and the plant dies.

Because they are sessile organisms, plants must deal biochemically with environmental stresses such as temperature extremes, nutrient deficiency and drought. This is also true for other photosynthetic organisms which are either sessile or limited in movement. Plants and other photosynthetic organisms, therefore, require signal transduction pathways in order to trigger cellular responses to adverse environmental stimuli.

It has long been known that both temporal and quantitative characteristics of flowering are affected by the level of phosphate in plants relative to the level of nitrogen (Salisbury and Ross, 1985). Relatively high phosphate advances maturity in plants, whereas relatively low phosphate results in little or no flowering taking place. Phosphate levels are also known to affect the biomass ratio between root and shoot. Specifically, phosphate deprivation causes preferential growth of roots (Lefebvre *et al.*, 1982). Thus, in many

environments, the availability of phosphorus becomes a major factor limiting the growth and reproduction of photosynthetic organisms.

Numerous groups have investigated the nature of the phosphate-starvation response in plants but despite these studies, little is known of the molecular mechanisms that regulate phosphorus uptake and metabolism. In general, plants exhibit significant morphological and physiological changes in response to perturbations within the environment.

There have been many attempts to identify proteins which are induced under conditions of phosphate starvation. Fife *et al.* (1990) have conducted *in vivo* protein labelling studies in *Brassica nigra* cells grown in suspension in either rich or low phosphate medium. Using 2-dimensional gel electrophoresis, they demonstrated the novel synthesis of four proteins under P_i deficiency and one protein in well-nourished cells. Other groups have reported that P_i deprivation increases the synthesis of a plasma membrane protein and a soluble protein in tomato root cultures (Hawkesford and Belcher, 1991), and enhances secretion of six proteins from tomato suspension cells (Goldstein *et al.*, 1989). It has also been shown that a gene for a protein homologous to β-glucosidases is induced to high levels in *B. nigra* suspension cells under P_i starvation (Malboobi and Lefebvre, 1995).

As part of the adenosine nucleotides, ADP and ATP, which are the currency of cellular energy, phosphorus is critical to bioenergetics. Further, the covalent addition or removal of a phosphate group to or from a biological substrate (phosphorylation and dephosphorylation, respectively) often functions as a

kind of regulatory "on/off switch" in cellular metabolism and signal transduction. For example, the phosphorylation and dephosphorylation of certain membrane-bound receptor protein kinases and their substrates are key to various signal transduction pathways, including pathways of plant hormones such as ethylene (Kieber *et al.*, 1993) and abscisic acid (Anderberg and Walker-Simmons, 1992). Self-incompatibility with respect to pollination and fertilization also involves the activity of protein kinases encoded by S-locus genes (Tantikanjana *et al.*, 1993; Zhang and Walker, 1993).

Knowledge of the proteins which affect the uptake and accumulation of phosphorus and which are expressed in phosphate-deficient environments is essential to understand phosphate metabolism and to manipulate the growth and reproduction of photosynthetic organisms for commercial or industrial purposes. Further, the identification and synthesis of the genes which encode such proteins would allow the development of transgenic photosynthetic organisms for many purposes.

Summary of the Invention

This invention provides the means to modify phosphorus metabolism in plants and other photosynthetic organisms by altering the expression and/or activity of one or more proteins involved in the response of plants or other photosynthetic organisms to phosphorus deprivation. This invention further provides means for more efficient metabolic utilization of phosphorus by plants and other photosynthetic organisms. The compounds of this invention provide the means to change plant morphology by altering phosphorus metabolism. In

some applications, the modification will be restricted to seeds, where it can lower the amount of phytate, an anti-nutritive phosphorus storage compound.

This invention relates to isolated DNA (genes) encoding proteins involved in phosphorus uptake and metabolism of plants and other photosynthetic organisms inducible by phosphate deficiency (*psr* proteins), as well as DNA complementary to these genes, and recombinant DNA constructs and vectors containing DNA encoding such proteins or such complementary DNA, in whole or portions thereof.

In particular, the present invention provides DNA (genes) encoding protein kinases, β-glucosidases, and phosphate transporters of *Arabidopsis thaliana* and *Brassica nigra*, whose transcription is inducible by phosphate starvation, and further provides the RNA so transcribed. The nucleic acids (both DNA and RNA) of this invention encode proteins which differ from other protein kinases in having a unique portion of their amino acid sequence which is different from any other known protein kinase. The β-glucosidases and phosphate transporters of this invention differ from other known β-glucosidases and phosphate transporters in sequence and because their level of expression is specifically dependent on phosphate deprivation.

Other nucleic acids of the invention include nucleic acids with sequences complementary to the nucleic acid sequences of *Arabidopsis thaliana* and *Brassica nigra*, or portions thereof; nucleic acids with sequences related to, but distinct from, the nucleic acid sequences of *Arabidopsis thaliana* and *Brassica nigra* and inducible under conditions of phosphate deficiency; and nucleic acid sequences that differ from

the nucleic acid sequences of *Arabidopsis thaliana* and *Brassica nigra*, such as modified analogs, due to alteration of the sequence through mutation, substitution, deletion and the like. Primers and probes consisting of 20 or more contiguous nucleotides of the above-described nucleic acids are also included as part of this invention. Homologues and other proteins which are similar in function and are *psr* proteins are also encompassed by this invention.

Thus, one type of nucleic acid of the invention is an antisense oligonucleotide, a triple helix-forming oligonucleotide, or other oligonucleotide that can be used to inhibit the expression of the *psr* proteins encoded by the nucleic acids described herein. Such oligonucleotides can block the expression of any of these proteins in a number of ways; for example, preventing transcription of a *psr* protein-encoding gene by triple helix formation, or by binding to the mRNA transcribed by the gene in any manner that prevents a functional protein from being assembled. Typically, and depending on the mode of action, the oligonucleotides of the invention comprise a specific sequence of about 20 to about 200 or more nucleotides which are identical or complementary to a specific sequence of nucleotides of the *psr* protein-encoding gene or transcribed mRNA.

The invention further provides nucleic acids of the invention operatively linked to a regulatory sequence, and plasmids or recombinant expression vectors for producing the nucleic acids encompassed by this invention. In a preferred embodiment, a recombinant expression vector, comprising the nucleic acids operatively linked to a regulatory sequence is adapted for transformation of a plant cell.

The invention also provides transformed or transgenic cells expressing one or more of the *psr* proteins of the invention. In a preferred embodiment, the transgenic cells are plant cells. The invention includes transgenic plants produced with nucleic acids or vectors of the invention which express *psr* proteins provided by the invention. The invention further includes transgenic plant parts, including seeds, as well as tissue culture or protoplasts produced with nucleic acids or vectors of the invention.

The invention also provides a recombinant expression vector adapted for transformation of a plant cell, comprising a DNA molecule operatively linked to a regulatory sequence to allow expression of an RNA molecule that is antisense to a nucleic acid sequence having substantial sequence homology with any of the nucleotide sequences represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, and SEQ ID NO:19 through SEQ ID NO:27.

The invention further provides a method of preparing a *psr* protein having *psr* protein activity using the nucleic acids of the invention. The method comprises culturing a transformant or transgenic cell including a recombinant expression vector comprising a nucleic acid of the invention and a regulatory sequence operatively linked to the nucleic acid in a suitable medium until the *psr* protein is expressed, and then isolating the *psr* protein. The invention also provides an isolated *psr* protein or polypeptide having *psr* protein activity and substantial sequence homology with either or both of the amino acid sequences shown in SEQ

ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:18 or a portion thereof.

Antibodies and antibody fragments which bind to the novel *psr* proteins described herein (or to portions of these sequences) are also included in this invention. In a preferred embodiment, the antibody is a monoclonal antibody.

The invention further provides a method for reducing expression of a *psr* protein of a photosynthetic organism, preferably a plant, comprising the step of incorporating into the organism an isolated nucleic acid which is antisense to a nucleic acid having substantial sequence homology with the nucleotide sequence of a gene encoding a *psr* protein, especially SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, and SEQ ID NO:19 through SEQ ID NO:27.

The methods of this invention can also be used to override dominant alleles thereby producing lowered expression rates or, at an extreme, the "null allele" phenotype. This occurs when a partial or complete complementary sequence to an mRNA is present in the cell. It is generally assumed that, in the presence of antisense RNA, mRNA:antisense hybrids are produced with the result that a substantial reduction in detectable levels of the target gene product is observed. The antisense transcripts can cause a reduction in steady-state sense mRNA levels, perhaps because of increased turnover, or specific duplex attack by double-stranded RNases (Murray and Crockett (1992)). The construction of antisense genes must take into consideration that expression levels have to be sufficiently high and be temporally coincident with target gene expression. In

addition, in order to avoid the possibility of affecting gene expression at loci of distinct functions, sequence specificity must be assured. This can be achieved by selecting fragments of the nucleic acid sequences encoding *psr* proteins from translated or from untranslated regions.

The invention further provides a method for reducing expression of a *psr* protein of a plant, comprising the step of incorporating into a plant, an isolated nucleic acid which causes co-suppression of genes which are identical to or which have substantial sequence homology to the nucleic acid sequences of *psr* proteins.

The invention further provides a method for lowering or increasing the activity of a *psr* protein of a plant, comprising the step of incorporating into the plant an isolated nucleic acid which causes the production of an altered *psr* protein such that it is either more active, or is dysfunctional and interferes with the native (naturally-occurring) functional *psr* protein in any way that its activity is reduced.

Thus, this invention provides means for regulating the response of a photosynthetic organism to varying levels of phosphate in its environment as well as a mechanism for modifying the phosphate metabolism of such organisms. This approach to modifying the phosphate pathways of plants has several advantages over traditional plant breeding methods, most importantly, the modifications can be made quickly and specific traits can be modified, even introducing a new trait which is not part of the plant genome.

Brief Description of the Figures

Figure 1 is a graph of the effect of different P_i treatments on endogenous phosphate content (open symbols) and dry weight accumulation (closed symbols) of *Brassica nigra* cells during 7 days of growth. Initial concentrations of P_i in the media were zero (o, ●), 1.25 mM (□, ■) and 10 mM (△, ▲).

Figure 2 is a densitometric scan of an autoradiogram of an SDS-polyacrylamide gel of ^{35}S-labelled *in vitro* translation products of poly(A)$^+$ RNA extracted from *B. nigra* suspension cells grown for 7 days in MS media containing either no P_i (dotted line), 1.25 mM P_i (dashed line) or 10 mM P_i (solid line). Panel A shows the high molecular weight region of the gel; panel B the medium molecular weight region; and panel C the low molecular weight region. Arrows indicate peaks corresponding to induced polypeptides. Estimated molecular weights are presented on the x-axis.

Figure 3 is a histogram showing the relative amounts of differentially expressed mRNA species in *B. nigra* suspension cells cultured in various concentrations of P_i. The length of each bar represents the area under the corresponding peak and, therefore, the relative abundance of the mRNA species. Peak designations are as in Figure 2. Estimated molecular weights are presented on the y-axis.

Figure 4 shows northern blots of total RNA extracted from 7-day old (a) minus P_i-treated, (b) 1.25 mM P_i-fed, and (c) 10 mM P_i-fed *B. nigra* suspension cells. *Tub A* is the α-tubulin gene that was used as a standard. Values given to the left of each panel are size estimates of mRNA species corresponding to the respective *psr* clones. Each lane contained 30 μg total RNA.

Figure 5 shows the DNA sequence (SEQ ID NO:1) of phosphate starvation-induced protein kinase *psr*PK (*psr1*) from *Arabidopsis thaliana* and the encoded amino acid sequence of the protein kinase (SEQ ID NO:2).

Figure 6 is a comparison of the cDNA sequence (SEQ ID NO:1) encoding the phosphate starvation-induced protein kinase *psr*PK (*psr1*) from *Arabidopsis thaliana* with the cDNA sequence (SEQ ID NO:3) encoding a homologous protein kinase from *Brassica nigra*. Boxed residues indicate conserved nucleotides between the two sequences.

Figure 7 shows the DNA sequence (SEQ ID NO:1) encoding the phosphate starvation-induced *psr*PK from *Arabidopsis thaliana* with its unique 3' terminal sequence capitalized and underlined.

Figure 8 is a comparison of the amino acid sequences of *Arabidopsis thaliana psr*PK (*psr*1) (SEQ ID NO:2) and *B. nigra psr*1 (SEQ ID NO:4) with the amino acid sequences of other protein kinases.

Figure 9 shows a computer analysis of *Arabidopsis thaliana psr*PK protein deduced amino acid sequence.

Figure 10 shows the results of a nuclear runoff experiment described in Example 7.

Figure 11 is a comparison of the 3' end of the cDNA sequences of *Arabidopsis thaliana psr*PK (*psr*1) and *B. nigra psr*1 with the 3' end of the DNA sequences of other protein kinases.

Figures 12A - 12D depict schematic representations of sense and antisense *psr*PK constructs in which a constitutive (CaMV-35S) or a seed-specific (Arabin-pro) promoter is fused with the sense (*psr*1) or antisense (α-*psr*1) *psr*PK genes.

Figure 13 is a map of the *Arabidopsis thaliana* clone (determined by Southern blotting of the restriction enzyme-digested DNA probed with the *B. nigra psr*3.1 cDNA) with the location of the *psr*3.2 indicated by an arrow.

Figures 14A and 14B show the DNA sequence (SEQ ID NO:5) of phosphate starvation-induced β-glucosidase (*psr*3.2) from *Arabidopsis thaliana* and its deduced amino acid sequence (SEQ ID NO:6)

Figure 15 is the nucleotide (SEQ ID NO:7) and deduced amino acid sequence (SEQ ID NO:8) of *Brassica nigra psr*3.1 cDNA clone (*psr*3.1B).

Figure 16 is the nucleotide (SEQ ID NO:9) and deduced amino acid sequence (SEQ ID NO:10) of *Arabidopsis thaliana psr*3.1 cDNA clone (*psr*3.1A).

Figures 17A and 17B show a comparison of the amino acid sequences of *Arabidopsis thaliana psr*3.2, *psr*3.1A, and *B. nigra psr*3.1B with the amino acid sequences of other plant β-glucosidases.

Figure 18 is the partial DNA sequence of *psr*2 (SEQ ID NO:11 and SEQ ID NO:12) from *Brassica nigra*.

Figure 19 is the partial DNA sequence of *psr*4 (SEQ ID NO:13 and SEQ ID NO:14) from *Brassica nigra*.

Figure 20 is the partial DNA sequence of *psr*5 (SEQ ID NO:15 and SEQ ID NO:16) from *Brassica nigra*.

Figure 21 is the DNA sequence (SEQ ID NO:17) and encoded amino acid sequence (SEQ ID N0:18) of *psr*6 from *Brassica nigra*.

Figure 22 is the partial DNA sequence of *psr*7 (SEQ ID NO:19 and SEQ ID NO:20) from *Brassica nigra*.

Figure 23 is the partial DNA sequence of *psr*8 (SEQ ID NO:21) from *Brassica nigra*.

Figure 24 is the partial DNA sequence of *psr*9 (SEQ ID NO:22 and SEQ ID NO:23) from *Brassica nigra*.

Figure 25 is the partial DNA sequence of *psr*10 (SEQ ID NO:24 and SEQ ID NO:25) from *Brassica nigra*.

Figure 26 is the partial DNA sequence of *psr*11 (SEQ ID NO:26 and SEQ ID NO:27) from *Brassica nigra*.

Figures 27A-27B show the Southern Blot analysis of *Arabidopsis* genomic DNA.

Figure 28 is a diagram of *Arabidopsis thaliana* transformation and production of subsequent generations.

Detailed Description of the Invention

The present invention provides novel methods for producing photosynthetic organisms, especially plants, the organisms so produced and methods of their use. This invention is based, in part, on the discovery that the transcription and expression of several proteins are induced in phosphate-starved cells of photosynthetic organisms. Thus, this invention provides isolated DNA encoding at least a functional portion of a protein (*psr* protein) of a photosynthetic organism in which transcription of the DNA is induced by phosphate deficiency. In particular, the genes encoding three classes of *psr* proteins, ser/thr (serine/threonine) protein kinases, β-glucosidases, and phosphate transporters, have been isolated and sequenced. As shown in the figures, all nucleic acids which encode *psr* polypeptides, and homologues of these *psr* nucleic acids, are encompassed by this invention.

Isolation of Clones

As an initial step in the investigation of the P_i-starvation response of *B. nigra* suspension cells, *in*

vitro translations of mRNA extracted from P_i-starved and P_i-fed cells were compared to investigate if alterations in protein synthesis profiles of *B. nigra* cells might be controlled at the transcriptional level. First, *B. nigra* suspension cells were grown in medium containing 1.25 mM P_i for 7 days, so that all cells would be in the same metabolic state. The cells were then subcultured into media with various initial concentrations of P_i. Growth conditions for the next 7 days were either severe P_i deprivation (0 P_i), mild P_i deprivation (1.25 mM P_i) or rich (10 mM P_i) (Lefebvre *et al.*,1990). In mild P_i deprivation, the plant cells absorbed all the P_i by day 2, whereas in the rich conditions, the cells did not take up all the P_i even after 7 days in culture. Total mRNA was isolated from each culture and these isolates were subjected to *in vitro* translation. The resultant polypeptides were separated on a high resolution SDS-PAGE gel.

Phosphate starvation did not cause gross changes in the protein synthesis profiles of *B. nigra* (Fife *et al.*, 1990). However, by comparison with the 10 mM P_i-fed cells, the inventors consistently isolated lower amounts of RNA from the minus P_i-treated cells, indicating a possible decrease in the rate of protein synthesis. This agrees with the high content of free amino acids observed in these cells (Duff *et al.*, 1994).

Scanning densitometry of the SDS-PAGE gel identified four polypeptides (approx. 31.7, 32.3, 52.5, and 64.8 kDa) present only in the P_i-starved samples (see Figure 2). These results agree with those of the *in vivo* protein synthesis analysis of *B. nigra* suspension cells reported by Fife, *et al.* (1990). Using 2-dimensional gel electrophoresis, they showed the novel

synthesis of four proteins (64 kDa, pI 5.2; 41 kDa, pI 5.6; 27 kDa, pI 5.7; 27 kDa, pI 5.2) under P_i deficiency and one protein (33 kDa, pI 5.1) in well-nourished cells. Acknowledging that such comparisons are at best speculative since post-translational modifications can only be made in living cells, the inducible proteins reported by Fife *et al.* possess similar sizes to those detected in this study.

A cDNA library was constructed from mRNA isolated from the severely deprived *B. nigra* cells. Screening by differential hybridization was performed on this cDNA library using cDNA probes prepared from minus P_i-treated and 10 mM P_i-fed (well-fed) *B. nigra* cells. A number of clones representing mRNA species preferentially transcribed under P_i-deficiency were identified. These phosphate-starvation responsive (*psr*) clones (121 clones) were placed into eleven different homology groups as determined by cross-hybridization. Northern blots showed that the expression of each of the eleven distinct groups of genes is controlled at the level of transcription (Malboobi and Lefebrve, 1995). The Northern blots showed that corresponding genes are inducible in both mild and severe P_i-starvation conditions; that is, possible side effects of extremely stressful conditions leading to cell death on the induction of these genes can be ruled out.

As shown in Figure 3, the expression of certain genes (grey bars) was also induced in the 7-day old 1.25 mM P_i-fed cells that had undergone mild P_i deprivation. The corresponding protein sizes are approximately 31.7, 32.3, 52.5 and 64.8 Kd. If *in vitro* transcription rates are independent of message type, then the levels of *de novo* expression of four of the differentially expressed

messages in minus P_i-treated cells were relatively high, comparable to those of the most abundant mRNAs in these cells. Based on expression patterns, the proteins encoded by these genes play active metabolic and structural roles in cellular adaptation to P_i stress.

Identification and Characterization of Phosphate-inducible Genes: Protein Kinase *psr* Genes

DNA sequencing and subsequent analysis permitted the identification of one of the genes, *psr*PK (*psr1*), as a protein kinase (SEQ ID NO:3) whose expression is induced in phosphate-starved *Brassica* nigra cells. A homologue (SEQ ID NO:1) which is also differentially expressed under P_i deprivation was identified and isolated from *A. thaliana*. The genomic clone of *psr*1 from *Arabidopsis* has also been obtained (Figures 27A-27B). For Southern Blot analysis, the DNA was digested with *Eco*RI (A), *Sac*I (B), *Sal*I (C), *Eco*RV (D), and *Bam*HI (E). The blots were probed with an 800 bp fragment representing the 3'-end of the *psr*I cDNA (Figure 27A), and the genomic clone isolated by screening a genomic library with this 800 bp fragment (Figure 27B). The common bands detected in both cases indicate that this genomic clone represents an allele of the *psr*I gene. Sizes of the bands hybridizing to the probes are indicated.

The induced *Arabidopsis thaliana* gene encodes a polypeptide designated *psr*PK (or *psr*1) which, along with the *B. nigra* polypeptide, has regions of high homology to other protein kinases (see Example 9 and Figure 8, *infra)*, and possesses serine/threonine (ser/thr) protein kinase activity. However, *A. thaliana psr*PK and its *B. nigra* homologue are different from previously described

protein kinases because they have a unique C-terminal region of the protein kinase. This unique region could be involved in P_i concentration detection or in receiving or delivering signals, or more than one of these functions. The protein kinases' substrates could be other components of the phosphate-starvation response pathway or enzymes involved in the response itself. These proteins have no apparent N-terminal signal peptide, organellar targeting sequence or membrane spanning regions, which indicates they probably function in the cytoplasm of the cell.

Protein kinases catalyze phosphorylation of protein substrates and are found in all living organisms. They are known to be involved in regulatory processes, wherein phosphorylation/dephosphorylation functions as a type of switch for the activation/deactivation (or vice versa) of the substrate protein. Certain types of protein kinases are involved in the phosphate starvation response of fungi and bacteria; however, this is the first time that a plant protein kinase has been shown to be inducible to high levels under P_i starvation. Because *psr*PK is particularly active during periods when phosphate is unavailable, it is probable that it has a switch-like role in the control of the plant response to phosphate deprivation. The *psr*PK protein is homologous to SNF1, which is expressed in carbon-starved bacteria and has been shown to be involved in governing metabolic reactions under such conditions. Thus, modulation of the expression of the *psr*PK kinase could alter the expression of whole pathways involved in phosphate metabolism, thereby producing valuable phenotypes.

Identification and Characterization of Phosphate-inducible Genes: β-glucosidase *psr* Genes

One homology group (*psr*3) of phosphate-starvation responsive cDNA clones from *Brassica nigra* was determined to contain a β-glucosidase based on a portion of the polypeptide sequence (Malboobi and Lefebvre, 1995). The DNA sequence (SEQ ID NO:7) encoding this phosphate starvation-induced β-glucosidase (*psr*3.1B) from *Brassica nigra* and the amino acid sequence (SEQ ID NO:8) of the full-length *psr*3.1B protein is shown in Figure 15.

Southern blots of *Arabidopsis thaliana* genomic DNA probed with the *psr*3.1 cDNA indicated that this gene exists as a single locus. A genomic library of *A. thaliana* was screened at high stringency to isolate the corresponding genomic clone. The resultant clone was designated *psr*3.2 (SEQ ID NO:5) because of its sequence divergence from isolated *psr*3.1 cDNA clones. Northern blotting with probes derived from the coding region of the genomic clone showed that this gene is expressed at high levels in P_i-starved roots and enhancement occurs within two days of growth in medium lacking P_i. The expression of this gene is repressed by heat shock and anaerobic conditions, and it is not significantly induced by high salinity, or by nitrogen or sulphur deprivation. Sequence analysis of the genomic clone revealed the existence of thirteen exons interrupted by twelve AT-rich introns and shows high homology with the *B. nigra psr*3.1B, as well as various other β-glucosidase genes from other species. Sequence similarity and divergence percentages between the deduced amino acid sequences of the *psr*3 clones and other β-glucosidases suggests that these genes should be included along with

two other Brassicaceae genes in a distinct subfamily of the BGA glycosidase gene family. The presence of an endoplasmic reticulum retention signal at the carboxy terminus indicates that this is the cellular location of *psr*3.2. The possible metabolic and regulatory roles of this enzyme during the P_i-starvation response are described *infra*.

Identification and Characterization of Phosphate-inducible Genes: Other Regulated *psr* Genes

DNA sequencing and computer analysis for the remaining nine *psr* clones was performed using the techniques described in Example 8. Genomic libraries of other species were screened as described in Example 9 to identify homologues of the genes.

The DNA sequence of *psr*2 from *Brassica nigra* most closely resembles DNA encoding glutamate dehydrogenase at the T3 portion of the sequence and an ovomucoid protein at the T7 portion of the sequence (Figure 18).

The DNA sequence of *psr*4 from *Brassica nigra* most closely resembles DNA encoding an envelope protein (Figure 19).

The DNA sequence of *psr*5 from *Brassica nigra* most closely resembles DNA encoding an aspartate kinase (Figure 20). This sequence shows some homology to DNA encoding disintegrin, another aspartate kinase, which inhibits the signal transduction pathway for the cell cycle.

The DNA sequence of *psr*6 from *Brassica nigra* and the encoded protein most closely resembles DNA encoding a phosphate transporter and the encoded protein (Figure 21).

The DNA sequence of *psr*7 from *Brassica nigra* most closely resembles DNA encoding a histidine kinase at the T3 portion of the sequence and a skeletal muscle calcium release channel protein at the T7 portion of the sequence (Figure 22).

The DNA sequence of *psr*8 from *Brassica nigra* (Figure 23) most closely resembles DNA encoding a sugar transporter protein.

The DNA sequence of *psr*9 from *Brassica nigra* most closely resembles DNA encoding an adenylate cyclase (Figure 24).

The DNA sequence of *psr*10 from *Brassica nigra* most closely resembles DNA encoding a calcium channel protein (G-protein) (Figure 25).

The DNA sequence of *psr*11 from *Brassica nigra* most closely resembles DNA encoding a phosphatidylinositol kinase at the T3 portion of the sequence and a tripeptidyl peptidase protein at the T7 portion of the sequence (Figure 26).

Isolated Nucleic Acids and Constructs

This invention provides isolated DNA or recombinant nucleic acids encoding a protein, or a functional portion thereof, of a photosynthetic organism wherein transcription (and/or translation) of the DNA or nucleic acid is induced by phosphate deficiency. In a preferred embodiment the protein has protein kinase activity, especially ser/thr protein kinase activity, or β-glucosidase activity or phosphate transporter activity. The term "nucleic acid" includes DNA and RNA, as well as single-stranded and double-stranded species.

DNA or nucleic acids referred to herein as "isolated" are DNA or nucleic acids separated away from

the nucleic acids of the genomic DNA or cellular RNA of their source or origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and may have undergone further processing. "Isolated" DNA or nucleic acids include DNA or nucleic acids obtained by methods described herein, similar methods or other suitable methods, including essentially pure DNA or nucleic acids, DNA or nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated. Nucleic acids referred to herein as "recombinant" are nucleic acids which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector using restriction enzymes. "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

The isolated DNA can comprise: (a) SEQ ID NO:1, SEQ ID NO:3, or a truncated nucleic acid sequence which encodes a functional portion of the protein encoded by SEQ ID NO:1 or SEQ ID NO:3 or (b) a nucleic acid sequence having at least 80% homology to SEQ ID NO:1, SEQ ID NO:3, or a truncated nucleic acid sequence which encodes a functional portion of the protein encoded by a nucleic acid sequence having at least 80% homology to SEQ ID NO:1 or SEQ ID NO:3; or (c) a nucleic acid which is complementary and hybridizes under moderately stringent conditions to any of the sequences of (a) or

(b). A "functional portion" means a portion of a *psr* protein which when expressed will affect the phosphate uptake and/or metabolism of the native (naturally-occurring) photosynthetic organism in which the endogenous *psr* protein is produced. A preferred embodiment is a truncated DNA sequence comprising isolated DNA consisting of nucleotide residues 677 to 1020 of SEQ ID NO:1, encoding the unique region of the protein kinase. Further provided is DNA or RNA having 50% homology, preferably 80% homology, or more preferably 90% homology, or which hybridizes under moderately stringent conditions to the DNA of Claim 3. Truncated nucleic acid sequences of the above-described DNA or nucleic acids which consist of 10-20 or more contiguous nucleotides are also provided and can find use as probes and primers.

One cDNA of this invention is shown in Figure 5 (SEQ ID NO:1) and comprises a 1020 nucleotide open reading frame, bounded by ATG start and TAG stop codons, encoding 339 amino acids. The cDNA further comprises 89 bp 5' untranslated nucleotides and 141 bp 3' untranslated nucleotides, including a 3' polyA tail for a functional mRNA. The protein encoded by this cDNA is discussed in detail below.

The isolated DNA further comprises: (a) SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, SEQ ID NO:19 through SEQ ID NO:27, or a truncated nucleic acid sequence which encodes a functional portion of the protein encoded by any of these sequences or (b) a nucleic acid sequence having at least 80% homology to SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, SEQ ID NO:19 through SEQ ID NO:27, or a truncated nucleic acid

sequence which encodes a functional portion of the protein encoded by a nucleic acid sequence having at least 80% homology to any of these sequences; or (c) a nucleic acid which is complementary and hybridizes under moderately stringent conditions to any of the sequences of (a) or (b). Polypeptides encoded by these nucleic acids are also encompassed by this invention. Further, an isolated nucleic acid encoding a protein having β-glucosidase activity and an amino acid sequence with at least 80% sequence homology with SEQ ID NO:5 or 50% homology with SEQ ID NO:6 is also provided, as is a nucleic acid encoding a protein having phosphate transporter activity and an amino acid sequence with at least 80% sequence homology with SEQ ID NO 17 or 50% homology with SEQ ID NO:18. Truncated nucleic acid sequences of the above-described DNA or nucleic acids which consist of 10-20 or more contiguous nucleotides are also provided and can find use as probes and primers.

For the purposes of this disclosure, the term "homology" does not refer to common evolutionary origin, but rather to similarity between sequences. The degree of homology between two sequences can be determined by optimally aligning the sequences for comparison, as is commonly known in the art, and comparing a position in the first sequence with a corresponding position in the second sequence. When the compared positions are occupied by the same nucleotide or amino acid, as the case may be, the two sequences are homologous at that position. The degree of homology between two sequences is expressed as a percentage representing the ratio of the number of matching or homologous positions in the two sequences to the total number of positions compared.

The term "having substantial sequence homology" is understood to mean that the sequence in question has slight or insignificant sequence variations from, for example, the sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:2 or SEQ ID NO:4. That is, a nucleic acid sequence "having substantial sequence homology" to SEQ ID NO:1 or SEQ ID NO:3 encodes substantially the same protein product as the actual sequence; *i.e.*, a protein kinase having a regulatory function in phosphate metabolism. It is expected that certain substitutions or other alterations will be able to be made in various portions of SEQ ID NO:1 or SEQ ID NO:3 which do not significantly affect protein function. The sequence variations may derive from mutation. Further, a protein having a homologous sequence to that of SEQ ID NO:2 or SEQ ID NO:4 would have a similar catalytic activity to that of the protein whose sequence is shown in SEQ ID NO:2 or SEQ ID NO:4. Alternatively, isoforms of the protein of SEQ ID NO:2 or SEQ ID NO:4 that have protein kinase activity could exist. For example, a sequence having substantial homology to that of SEQ ID NO:2 can be a homologue from another plant variety or species, as is SEQ ID NO:4. Such isoforms and homologous proteins may be immunologically cross-reactive.

It is expected that a nucleic acid encoding a protein comprising an amino acid sequence having about 80% homology with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or about 50% homology with the amino acid residues 190 to 340 of SEQ ID NO:2, will produce a functional protein kinase, and the invention provides such a nucleic acid. Proteins comprising an amino acid sequence that is about 60%, 70%, 80% or 90% homologous with the amino acid sequence of SEQ ID NO:2 or SEQ ID

NO:4, or greater, are also expected to have protein kinase activity.

The invention encompasses isolated nucleic acids encoding a protein having protein kinase activity, and having a sequence that differs from the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3 due to degeneracy in the genetic code. "Degeneracy" is understood to mean that each of several different amino acids is designated by more than one nucleotide triplet or codon. For example, AAA and AAG each code for lysine. This is an example of a "silent mutation" occurring in the third (or "wobble") nucleotide of a codon wherein the amino acid encoded remains the same. The invention also encompasses mutations that are not silent or other alterations wherein at least 80% amino acid homology with SEQ ID NO:2 or SEQ ID NO:4, or at least 50% homology with amino acid residues 190 to 340 of SEQ ID NO:2, is maintained.

Thus, nucleic acid and amino acid sequences having substantial sequence homology to any of SEQ ID NO:5 through SEQ ID NO:27 are also encompassed by this invention.

It would be understood by a person skilled in the art that the invention includes different forms of the nucleic acids of the invention arising from alternative splicing of an mRNA corresponding to a cDNA of the invention.

The invention further provides a nucleic acid that hybridizes under high or moderate stringency conditions to a nucleic acid encoding at least a portion of the amino acid sequence shown in SEQ ID NO:2 or SEQ ID NO:4, or other *psr* proteins or polypeptides. Stringency

conditions for hybridization is a term of art which refers to the conditions of temperature and buffer concentration which permit hybridization of a particular nucleic acid to another nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree of complementarity which is less than perfect. See, sections 2 and 6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 1, Supplement 29, 1995). Hybridization conditions are described generally in Maniatis *et al.*, 1982 and Sambrook *et al.*, 1989.

High stringency hybridization procedures, for example, can (1) employ low ionic strength and high temperature for washing, such as 0.015 M NaCL/0.0015 M sodium citrate, pH 7.0 (0.1x SSC) with 0.1% sodium dodecyl sulfate (SDS) at 50°C; (2) employ during hybridization, 50% (vol/vol) formamide with 5x Denhardt's solution (0.1% weight/volume highly purified bovine serum albumin/0.1% wt/vol Ficoll/0.1% wt/vol polyvinylpyrrolidone), 50 mM sodium phosphate buffer at pH 6.5 and 5x SSC at 42°C; or (3) employ hybridization with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2x SSC and 0.1% SDS.

By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize with the most similar sequences in the sample can be determined.

This invention also provides nucleic acids and polypeptides with structures that have been altered by different means, including but not limited to, alterations using transposons, site-specific and random mutagenesis, and engineered nucleotide substitution, deletion, or addition.

Proteins

This invention also relates to *psr* proteins or *psr* polypeptides, for example the proteins encoded by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, SEQ ID NO:19 through SEQ ID NO:27, or by homologous nucleic acids. As shown in the figures, all *psr* polypeptides and homologues are encompassed by this invention.

The proteins and polypeptides of the present invention can be isolated and/or recombinant. Proteins or polypeptides referred to herein as "isolated" are proteins or polypeptides purified to a state beyond the naturally-occurring state in which they exist endogenously in cells. A preferred embodiment is an essentially pure protein or polypeptide free of other proteins or polypeptides. "Isolated" proteins or polypeptides include proteins or polypeptides obtained by methods described herein, similar methods or other suitable methods, including essentially pure proteins or polypeptides, proteins or polypeptides produced by chemical synthesis, or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Proteins or polypeptides referred to herein as "recombinant" are proteins or polypeptides produced by the expression of recombinant nucleic acids.

In their native state, the transcription of these proteins is induced under conditions of phosphate deficiency and the proteins have varying activities which affect levels and kinds of phosphate content in cells, such as protein kinase, β-glucosidase, and phosphate transporter activity. Preferably, the amino acid sequence of these proteins shows at least 80% sequence homology with SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:18 or 50% homology with amino acid residues 190 to 340 of SEQ ID NO:2.

Isolation of Homologues

Nucleic acids or portions thereof provided by this invention can be used to isolate homologous nucleic acids from cells of other species of photosynthetic organisms which contain genes encoding one or more *psr* proteins similar in function to the nucleic acids of this invention. The term "*psr* protein" means a protein whose expression is induced to higher levels under conditions of phosphate deficiency than under conditions of phosphate sufficiency. The protein may not be expressed at all if the cell has adequate levels of available phosphate. Further such a protein is not associated with mechanisms which bring about cell death.

As described in the examples below, the inventors have isolated nucleic acids of the invention using *B. nigra* suspension cells as starting material. In brief, the inventors created a cDNA library from *B. nigra* suspension cells that had been starved for phosphate for seven days. The library was screened using two sets of cDNA probes generated from *B. nigra* suspension cells that had been grown under conditions of no phosphate or

5 mM phosphate, respectively. Clones that hybridized strongly to the first set of probes, but not the second set, were isolated. The cDNA inserts of these clones were subjected to dideoxy nucleotide sequencing (Sanger, 1981) to determine nucleotide sequence, and amino acid sequence was predicted therefrom. The *psr*PK gene was recognized to encode a novel protein kinase. Other differential hybridization, cloning and sequencing methods are known to those skilled in the art, and can be employed to obtain the protein kinase genes isolated by the inventors, other *psr* genes, or homologues thereof.

Alternatively, a nucleic acid of the invention can be isolated in the following manner. A nucleic acid probe comprising at least a portion of the sequence of SEQ ID NO:1 (or another *psr* sequence), or a homologue is chemically synthesized or prepared using recombinant DNA techniques. The probe is radiolabelled and used to screen a cDNA or genomic DNA library according to standard techniques. It can be prepared from *B. nigra*, a different organism or another source having a homologue or transgene of the invention that could be identified under appropriate hybridization conditions. The DNA identified by screening the library is then cloned and sequenced using standard techniques.

A third alternative method for isolating a nucleic acid of the invention is to isolate or chemically synthesize a peptide of the protein kinase (or another *psr* gene) and use this peptide to produce an antibody to the encoded *psr* protein in an animal: The antibody is then used according to standard techniques to screen a cDNA library, from *B. nigra* or another source, for

immunoreactive clones. DNA from such clones are then sequenced as is known in the art.

A fourth alternative method for isolating a nucleic acid of the invention is to selectively amplify such a nucleic acid using polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Konat *et al.*, 1991) and DNA or RNA as a template. In the latter case, total mRNA may be isolated from cells using one of the methods common in the art, described in Maniatis *et al.*, 1982. The retroviral enzyme reverse transcriptase is then used to synthesize cDNA complementary to the mRNA. Appropriate oligonucleotide primers for the amplification of the chosen nucleic acid are designed and synthesized, and PCR performed on a mixture of the primers and cDNA using standard technology (Innis *et al.*, 1990). The PCR protocol can additionally include 5' or 3' RACE (rapid amplification of cDNA ends) methodology (Innis *et al.*, 1990). The amplified DNA fragment produced is cloned into an appropriate vector.

An RNA molecule of the invention can also be constructed by cloning an appropriate cDNA or an amplified DNA molecule as described above into one of the commonly available transcription vectors. The DNA would usually be cloned downstream of a promoter, for example, the SP6 promoter of the vector pGEM 3Z (Promega, Madison, WI), the appropriate RNA polymerase (in this example, SP6 polymerase) added, and transcription reactions performed according to the manufacturer's specifications. Other useful promoters carried by commonly used transcription vectors are the bacteriophage T7 and T3 promoters.

Another well-known method of producing a nucleic acid or oligonucleotide of the invention is chemical

synthesis. Various machines for DNA synthesis are well-known in the art, such as, for example, those sold by Applied Biosystems, Inc. of Foster City, CA and by Millipore Corp. of Bedford, MA, and can be used for such syntheses.

Alteration of Protein Expression with Recombinant Expression Vectors

Alteration of *psr*PK or other *psr* protein expression is achieved in a variety of ways. In one embodiment, recombinant nucleic acids are constructed in which *psr*PK or another *psr* protein is operatively linked to regulatory sequences, such as promoters, that control the level, timing or tissue-specificity of gene expression.

Standard recombinant DNA techniques can be employed to engineer a recombinant expression vector including a nucleic acid of the invention that allows expression of at least a portion of a protein kinase or other *psr* protein of the invention. See, *e.g.*, Sambrook, *et al.*, 1989. The engineered vector would also include a regulatory sequence "operatively linked" to the nucleic acid of the invention to allow such expression. For the purposes of this disclosure, the term "regulatory sequence" includes promoters, enhancers and other sequences that control expression or message stability, as are well-known in the art. Examples of known promoters suitable for these purposes are given *infra*. Those of skill in the art can recognize that these examples are not limiting and other promoters can be adapted for particular purposes of modulating the phosphate uptake and metabolism of photosynthetic organisms.

In some cases, the regulatory element can provide tissue-specific expression. The two-part term "operatively linked" means both that the regulatory sequence contains sufficient element(s) to allow expression of the nucleic acid in question and that the nucleic acid is linked to the regulatory sequence appropriately. For example, the nucleic acid of the invention is in the appropriate orientation and in phase with an initiation codon.

In the context of this disclosure, the term "promoter" or "promoter region" refers to a sequence of DNA, usually upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing recognition and binding sites for RNA polymerase and/or other factors required for transcription to start at the correct site.

There are generally two types of promoters, constitutive and inducible promoters. The term "constitutive" as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types, although some variation in abundance is often detected.

An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically a protein factor (or factors), that binds specifically to an inducible promoter to activate transcription, is present in an inactive form which is then directly or indirectly converted to an active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite,

growth regulator, herbicide or phenolic compound, or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. The inducer can also be an illumination agent such as light, darkness and light's various aspects which include wavelength, intensity, fluence, direction and duration. A cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. If it is desirable to activate the expression of a gene at a particular time during plant development, the inducer can be so applied at that time.

Examples of such inducible promoters include heat shock promoters, such as the inducible hsp70 heat shock promoter of *Drosphilia melanogaster* (Freeling, *et al.*, 1985; a cold inducible promoter, such as the cold inducible promoter from *B. napus* (White, *et al.*, 1994,); and the alcohol dehydrogenase promoter which is induced by ethanol (Nagao, *et al.*, 1986).

Among sequences known to be useful in providing for constitutive gene expression are regulatory regions associated with *Agrobacterium* genes, such as nopaline synthase (Nos), mannopine synthase (Mas) or octopine synthase (Ocs), as well as regions regulating the expression of viral genes such as the 35S and 19S regions of cauliflower mosaic virus (CaMV) (Brisson, *et al.* 1984), or the coat promoter of TMV (Takamatsu, *et al.*, 1987).

Other useful plant promoters include promoters which are highly expressed in phloem and vascular tissue of plants such as the glutamine synthase promoter (Edwards, *et al.*, 1990) the maize sucrose synthetase 1

promoter (Yang *et al.*, 1990), the promoter from the Rol-C gene of the TLDNA of Ri plasmid (Sagaya, *et al.*, 1989), and the phloem-specific region of the pRVC-S-3A promoter (Aoyagi, *et al.*, 1988). Alternatively, plant promoters such as the small subunit of Rubisco (Rbcs) promoter (Coruzzi, *et al.*, 1984; Broglie, *et al.*, 1984), or heat shock promoters, e.g., soybean HPS17.5-E or HPS17.3-B (Gurley, *et al.*, 1986) can be used.

Other promoters which can be used according to the present invention include, but are not limited to:

(a) low temperature and ABA-responsive promoters, such as Kin1, cor6.6 (Wang *et al.*, 1995; Wang and Cutler, 1995) and the ABA inducible promoter from EM gene wheat (Marcotte Jr. *et al.*, 1989).

(b) phloem-specific sucrose synthase promoters, such as the ASUS1 promoter from *Arabidopsis* (Martin *et al.*, 1993);

(c) root and shoot promoters, such as the ACS1 promoter (Rodrigues-Pousada *et al.*, 1993);

(d) seed-specific promoters, such as the 22 kDa zein protein from maize (Unger *et al.*, 1993) the ps1 lectin promoter from pea (de Pater *et al.*, 1993), the phaseolin promoter from *Phaseolus vulgaris* (Frisch *et al.*, 1995);

(e) late embryo-abundant promoters, such as the lea promoter (T.L. Thomas, 1993);

(f) fruit-specific promoters, such as the E8 gene promoter from tomato, (Cordes *et al.*, 1989);

(g) meristematic tissue-specific promoters such as the

PCNA promoter (Kosugi *et al.*, 1995);

(h) pollen-specific promoters, such as the NTP303 promoter (Weterings *et al.*, 1995);

(i) late embryogenesis stage-specific promoters, such as the OSEM promoter (Hattori *et al.*, 1995);

(j) ADP-glucose pyrophosphorylase tissue-specific promoters for guard cells and tuber parenchyma cells, such as the ADP GP from potato (Muller-Rober *et al.*, 1994);

(k) conductive tissue-specific promoters, such as the Myb promoter from barley (Wissenbach *et al.*, 1993); and

(l) Plastocyanin promoters in young green tissues, such as the plastocyanin promoter from *Arabidopsis* (Vorst *et al.*, 1993).

Depending on the type of regulatory sequence employed, a plant transformed with a recombinant nucleic acid of this invention would over- or under-express a *psr* protein, either in chosen plant parts or throughout the plant, and/or at different times in the life history of the plant. Changes in plant size, relative sizes of different plant parts, time of flowering, level of phytate, starch and oil accumulated in seeds, or other phenotypic characteristics can thus be engineered.

Numerous recombinant expression vectors are known that are suitable for expression in a variety of cell types. A recombinant expression vector can be engineered for expression of a *psr* protein, such as *psr*PK, in prokaryotic cells, for example, *Escherichia coli*, or in eukaryotic cells, for example, *Saccharomyces cerevisiae* (yeast) and *Arabidopsis thaliana*, tobacco or canola. The recombinant expression vector can be a plasmid, a bacteriophage or a virus. Plant gene constructs of the present invention can be introduced using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection,

electroporation, and the like, as described, *supra*. Common expression vectors often include a marker gene that permits easy screening for transformed cells. Some common vectors also include a sequence encoding at least a portion of another functional protein, such as firefly luciferase or bacterial β-galactosidase. In an example of a scheme employing this kind of vector, a nucleic acid of the invention would be linked in frame to this coding sequence such that a fusion protein would be produced comprising at least a portion of the protein kinase of the invention and the other functional protein. Cells transformed with the engineered vector can be screened for expression of the luciferase, β-galactosidase or other fused protein. Alternatively, the other protein fused to the *psr* protein may not be useful for screening, but can instead provide a useful property, such as increased solubility, or can be exploited in a protein purification scheme or in industrial applications such as the addition of purified enzyme to a reaction.

Accordingly, the vectors of this invention can be constructed containing nucleic acids encoding a *psr* protein with which to transform a wide variety of crop and horticultural plants, including monocots, dicots and gymnosperms. Modification can be targeted to the whole plant, or to a specific tissue, organ or plant part, such as a seed. Further, expression of the gene can be limited to particular developmental stages or environmental conditions. The gene delivery systems used to incorporate the constructs will vary depending on the target plant species; however, those of skill in the art can recognize that present molecular techniques can be applied to successfully modify particularly

useful crop plants, such as rice, wheat, barley, rye, corn, soybeans, canola, sunflower, oranges, grapefruit, lemons, limes, potato, carrots, sweet potato, beans, peas, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, pepper, carrots, pumpkins, cucumber, apples, pears, melons, plum, cherry, peaches, nectarines, apricot, strawberry, grape, raspberry, pineapple, tobacco, bananas, sorghum, sugarcane, and the like.

For example, several plant species, canola, tobacco and *Arabidopsis*, have been genetically engineered to include vectors designed both to lower and to increase the amount of protein kinase within the entire plant or in the developing seeds.

In one embodiment, eight different constructs were incorporated into *Arabidopsis* cells with either constitutive (CaMV 35S) or seed-specific (Arabin) promoters. These constructs are described **in general** in Table 1 below.

Table 1

Construct	Promoter	*A. thaliana* protein kinase	sense/ antisense
1	Constitutive	complete gene sequence	sense
2	Seed-specific	complete gene sequence	sense
3	Constitutive	complete gene sequence	antisense
4	Seed-specific	complete gene sequence	antisense
5	Constitutive	truncated gene sequence	sense
6	Seed-specific	truncated gene sequence	sense
7	Constitutive	truncated gene sequence	antisense
8	Seed-specific	truncated gene sequence	antisense

The first four of these constructs containing complete gene sequence are depicted in Figures 12A-12D.

More specifically, *Arabidopsis thaliana* has been transformed using the constructs described in Table 2.

PSR1 Constructs and Transformation Progress

	CONSTRUCT	CONFIRMED?	Transformation of a..t. (T1) (-> T2 seeds)	Selection of T2 (-> T3 seeds)
C1	CaMV+sense *psr1*	yes	A, B	A:4, B:6
C7	CaMV+antisense *psr1*	yes	A, B	A:10, B:1
D1	Arabin+sense *psr1*	yes	A, B	A:4, B:14
D4	Arabin+antisense *psr1*	yes	A, B	A:4, B:0
#2	CaMV+*psr1* with mutated ATP binding site	yes	A	
#6	CaMV+*psr1* with mutated kinase active site	yes	A	
#26	CaMV+*psr1* with deleted Lys in ATP binding site	yes	A	
psr1	CaMV+ sense *psr1*	yes	A	
PA1	CaMV+antisense 3' *psr1*			
PA3	CaMV+antisense 3' *psr1*			
AA5	Arabin+antisense 3' *psr1*			
#2(P1+P2)	CaMV+*psr1* with point mutation at Thr 250 (phosphorylation site)	yes	A	
#12(P3+P4)	CaMV+*psr1* with point mutation at Ser 316 (phosphorylation site)	yes	A	

Description of Constructs and Terms shown in Table 2:

C1 full length sense *psr1* in pBI121 with cauliflower mosaic virus 35S promoter (CaMV 35S)

C7 full length anti-sense *psr1* in pBI121 with CaMV 35S promoter

D1 full length sense *psr1* in pBI121 with seed specific arabin promoter

D4 full length anti-sense *psr1* in pBI121 with seed specific arabin promoter

No.2 full length *psr1* with a mutated ATP binding site in pBI121 with CaMV 35S promoter (Lys changed to Glu at amino acid 33)

No.26 full length *psr1* with deleted Lys (amino acid 33) in ATP binding site in pBI121 with CaMV 35S promoter

No.6 full length *psr1* with mutated kinase active site in pBI121 with CaMV 35S promoter (Asp changed to Glu at amino acid 123).

psr1 full length *psr1* in pBI121 with CaMV 35S promoter (identical to C1).

3' *psr1* 175 bp unique 3' sequence

Nos. 2, 26, 6 and *psr1*: the inserts were between the promoter and the GUS gene

Transformation: 170 plants / construct/ transformation experiment

transformation experiments: A, B,...

Transformant cells

The invention provides host cells transformed with a recombinant expression vector of the invention. For the purposes of this disclosure, the terms "transformed with", "transformant", "transformation", "transfect with", "transfectant" and "transfection" all refer to the introduction of a nucleic acid into a cell by one of the numerous methods known to persons skilled in the art. Transformation of prokaryotic cells, for example, is commonly achieved by treating the cells with calcium chloride so as to render them "competent" to take up exogenous DNA, and then mixing such DNA with the competent cells. Prokaryotic cells can also be infected with a recombinant bacteriophage vector.

Nucleic acids can be introduced into cells of higher organisms by viral infection, bacteria-mediated transfer (*e.g.*, *Agrobacterium* T-DNA delivery system), electroporation, calcium phosphate co-precipitation,

microinjection, lipofection, bombardment with nucleic-acid coated particles or other techniques, depending on the particular cell type. For grasses such as corn and sorghum, microprojectile bombardment as described, for example, in Sanford, J.C., *et al.*, U.S. Patent No. 5,100,792 (1992) can be used. Other useful protocols for the transformation of plant cells are provided in Gelvin *et al.*, 1992. Suitable protocols for transforming and transfecting cells are also found in Sambrook *et al.*, 1989. The nucleic acid constructs of this invention can also be incorporated into specific plant parts such as those described *infra* through the transformation and transfection techniques described herein.

To aid in identification of transformed plant cells, the constructs of this invention are further manipulated to include genes coding for plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, or the like. In the constructs of Figures 12A-12D and those described in Table 2, the NOS/NPTII kanamycin-resistant gene is used to detect transfected plant cells. Similarly, enzymes providing for production of a compound identifiable by color change such as GUS (β-glucuronidase), or by luminescence, such as luciferase, are useful.

The invention provides transformant cells in which the introduced DNA has integrated into the genome, and transformant cells in which the introduced DNA exists as an extrachromosomal element. In the latter case, maintenance of an extrachromosomal element can be easily obtained by including a selectable marker in the recombinant expression vector and then, after

introduction of the vector, growing the cells under conditions where expression of the marker gene is required. In one embodiment of this invention, cells transformed with a recombinant expression vector of the invention are screened using protein kinase activity as a selectable marker. Such transformed cells can be screened, for example, under conditions of phosphate starvation.

The experiments depicted in Table 2 provide transformants of *Arabidopsis thaliana* selected by kanamycin resistance as shown in Figure 28 through several generations. T2 generation seed from initially transformed plants has been obtained from constructs Nos. 2, 6, and 26, and from construct *psr*1. T3 generation seed from 10, 11, 18 and 4 kanamycin-resistant plants has been obtained from constructs C1, C7, D1, and D4, respectively.

Plants and other photosynthetic organisms containing the nucleic acid constructs described herein are further provided by this invention. The term "photosynthetic organism" is meant to include the members of the kingdom Planta, including vascular and nonvascular plants (angiosperms, gymnosperms, ferns, mosses, bryophytes, etc.), the algae, photosynthetic protists (single-celled eukaryotes), the Cyanophyta (blue-green algae or cyanobacteria) and the photosynthetic bacteria. Suitable plants include both monocotyledons and dicotyledons. Examples of preferred monocotyledons are commercially-important crop plants such as rice, corn, wheat, rye, sugarcane and sorghum. Examples of preferred dicotyledons are canola, sunflower, citrus, tomato, broccoli, and lettuce. Algae can be used as a hosts for the constructs described

herein. Examples of such algae are *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Euglena gracilis*, *Porphyra purpurea*, *Cryptomonas* sp., and *Ochromonas sinensis*. Prokaryotes can also provide suitable host cells. Specific examples include *Anacystis nidulans*, *Synechococcus sp.*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Chloroflexus aurantiacus*, and *Heliobacterium chlorum*.

The constructs of the present invention can be introduced into plants, plant parts, or other cells of photosynthetic organisms using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see, for example, Weissbach and Weissbach, (1988) and Grierson and Corey, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9 (1988).

The method of obtaining transformed cells and/or regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which can contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Selected transformed plant cells can be induced to form callus tissue. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants.

The transformants or descendants of transgenic plants so produced can be evaluated with respect to growth and productivity. Transgenic plants can also be assessed for flowering characteristics, root and shoot size ratios, and seed phytate, starch and oil content.

The transformants would be used directly or could be subjected to further modifications by genetic engineering or classical techniques.

Transgenic plants containing the constructs of this invention can also be regenerated from plant tissues, plant parts, or protoplasts by methods known to those of skill in the art and as shown in Figure 28. Plant part is meant to include any portion of a plant capable of producing a regenerated plant. Thus, this invention encompasses cells, tissue (especially meristematic and/or embryonic tissue), protoplasts, epicotyls, hypocotyls, cotyledons, cotyledonary nodes, pollen, ovules, stems, roots, leaves, and the like. Plants can also be regenerated from explants. Methods will vary according to the plant species.

Seed can be obtained from the regenerated plant or from a cross between the regenerated plant and a suitable plant of the same species. Alternatively, the plant can be vegetatively propagated by culturing plant parts under conditions suitable for the regeneration of such plant parts. The plants can then be used to establish repetitive generations containing an altered genotype with respect to phosphate-inducible *psr* protein activity, either from seeds or using vegetative propagation techniques (see, *e.g.*, Figure 28).

Homology-dependent Gene Silencing including Antisense Nucleic Acids

The invention further encompasses homology-dependent gene silencing including silencing for the nucleic acid of the invention mediated by DNA-DNA pairing and by RNA. In the former, DNA interaction between an introduced nucleic acid or oligonucleotide

and the native homologue(s) result in co-suppression of the genes. RNA-mediated silencing includes antisense technologies in which a nucleic acid or oligonucleotide that is antisense to a nucleic acid of the invention is introduced into cells. Such an antisense molecule is capable of base-pairing (hydrogen bonding) with the nucleic acid of the invention in an anti-parallel manner, according to the standard pair rules, *i.e.*, G pairs with C, and A pairs with T or U. The antisense molecule can be complementary to a coding or non-coding region of a nucleic acid of the invention, including a non-coding regulatory region, or to portions of both (Gogarten *et al.*, 1992; Shotkoski and Fallon, 1994). The region of complementarity can precede or span the first codon of SEQ ID NO:1. An antisense molecule according to the invention can include a region complementary to a regulatory sequence, for example a non-coding regulatory sequence that is operatively linked to a gene of the invention in an expression construct. Alternatively, catalytic antisense RNA directed at the *psr* protein gene transcript (a ribozyme) can be employed to reduce gene expression (Heinrich *et al.*, 1993; de Feyter *et al.*, 1996). The antisense molecule can be produced by chemical synthesis, PCR or an expression vector, using certain of the techniques discussed in the previous section, or by other standard techniques. (Meyer, P. and Saedler, H., 1996).

Antisense constructs include, but may not be limited to, the following:

1. A whole *psr* gene placed in reverse orientation in respect to a promoter;

2. An antisense sequence complementary to the unique 3' region (nucleotides 930 through 1272) of Figure 7;

3. The first one-third of the antisense sequence described in (2) above (nucleotides 930 through 1080) which includes the partially conserved nucleic acid sequence encoding the EEEXXD sequence;

4. The second one-third of the antisense sequence described in (2) above (from nucleotide 1073) which includes the codon for the conserved "D" residue at position 336 of the amino acid sequence of Figure 8;

5. An antisense sequence complementary to the 3'-untranslated region up to the polyA tail (nucleotides 1008-1240); or

6. An antisense sequence complementary to the 5'-untranslated region (nucleotides 10 through 88).

If cell death occurs with an antisense construct under the control of a seed specific or constitutive promoter, an inducible promoter could be used. Sense or antisense nucleic acid according to the invention can be delivered to cells using any of a variety of methods known to persons skilled in the art.

Isolated Proteins

This invention provides an isolated protein having protein kinase, β-glucosidase, phosphate transporter, or other *psr* protein activity and, in particular, an amino acid sequence with substantial sequence homology with the amino acid sequence shown as SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:18. Alternatively, an isolated protein of the invention can be encoded by a nucleic acid that

hybridizes under low or high stringency conditions to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 through SEQ ID NO:17, SEQ ID NO:19 through SEQ ID NO:27, or a protein encoded by any *psr* gene. It should be understood that the invention includes protein fragments demonstrating such homology and having *psr* protein activity (*i.e.*, functional portions).

The mature, unmodified protein having the amino acid sequence shown as SEQ ID NO:2 is predicted to have a molecular weight of 39,040 kDa. Prosite searches were used to determine the following characteristics of the PSRPK protein. It contains ser/thr protein kinase active site between amino acid positions 119-131, and an ATP-binding site between positions 9-33. An hydrophobicity plot of the protein does not indicate any long regions of membrane associated protein and an antigenicity plot of the protein indicates several areas that would be appropriate for employment as peptides for antibody production against the protein. These include but are not restricted to the last 150 amino acids at the C-terminus. As there are no apparent N-terminal signal peptide or organellar targeting sequences, the protein appears to be cytoplasmically localized. There are a number of potential phosphorylation sites which include, but may not be restricted to, positions that are indicated by motifs for PKC, CK2, and tyrosine kinase phosphorylation sites as shown in Figure 9. These may or may not be autophosphorylation sites. There is a putative glycosylation site in the C-terminal region.

A *psr* protein of the invention can be purified from cells or from culture medium into which it has been

secreted. Alternatively, it can be chemically synthesized, as is well-known in the art. As discussed above, for the purposes of this disclosure, the term "isolated" means that the protein is substantially free of other cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when produced by chemical synthesis.

The invention further provides a method of preparing a protein having protein kinase activity, β-glucosidase activity, phosphate transporter activity, or other *psr* protein activity, or a fragment of such a protein, which includes the following steps: (i) transforming cells with a recombinant expression vector including a nucleic acid of the invention and a regulatory sequence operatively linked to the nucleic acid, (ii) culturing the transformant cells in a suitable medium until the *psr* protein is formed, and (iii) isolating the *psr* protein. A person skilled in the art would be able to devise a scheme for the isolation of the protein from other cellular material and culture medium using conventional techniques (Scopes, 1982). These include chromatographic methods such as gel filtration, ion-exchange chromatography and affinity chromatography, as well as batch methods employing ion-exchange or affinity resins. Precipitation with ammonium sulfate, followed by resuspension and dialysis is another common purification and concentration protocol.

In another embodiment of the invention, a fusion protein comprising at least a functional portion of the *psr* protein of the invention can be prepared by the method whose steps are detailed above. In some

embodiments, the *psr* protein or functional portion thereof can be fused to a signal sequence which directs secretion of the fusion protein from the transformant cells. The secreted protein can be isolated using standard techniques.

Antibodies

The invention encompasses an antibody that is specific for a *psr* protein of this invention. The invention includes both polyclonal and monoclonal antibodies. For purposes of this disclosure, the term "antibody" includes antibody fragments that are specific for a *psr* protein, such as a protein kinase, β-glucosidase, or phosphate transporter as described herein. Such fragments include F_{ab} fragments generated by proteolysis of an antibody. In some embodiments, an antibody can be directed to an epitope unique to the *psr* protein of the invention. If the protein is a protein kinase of this invention, for example, these can include the protein kinase active site and the ATP-binding site as well as the unique C-terminus sequence of 150 amino acids.

Intact *psr* proteins or an immunogenic fragment thereof can be used to prepare antibodies. The protein or fragment chosen as the antigen can be injected into an animal (e.g., rabbit, hamster, goat, mouse), causing the animal to produce antibodies specific to the injected antigen. The antigen is often combined with an adjuvant, such as Freund's adjuvant. In some cases, prior to injection, the antigen is conjugated to a hapten, or carrier molecule. A person skilled in the art would be aware of appropriate antigen dosages for the size and species of animal, how to design a schedule of repeated

injections if required, and how to titer and purify the antibody raised in the animal's serum. Methods of preparing antibodies including these and other aspects are described in Harlow and Lane, 1988.

For the production of monoclonal antibodies, lymphocytes raised against the antigen are first harvested, and then fused with myeloma cells using standard procedures (Harlow and Lane, 1988). The immortalized hybridoma cells so produced are screened for *psr* protein-specific antibodies using conventional immunoassay methods such as ELISA (enzyme-linked immunosorbent assay). The antibodies can then be purified as is known in the art.

An antibody of the invention can be physically coupled to any of a number of detectable substances that are known in the art. These include: a radioisotope, a fluorescent molecule, and an enzyme capable of catalyzing a colorimetric reaction. Examples of such an enzyme include alkaline phosphatase and horseradish peroxidase, which are commonly used in laboratory assays.

Thus, this invention provides a method of detecting the expression of a phosphate-starvation inducible protein, such as a protein kinase, a β-glucosidase, or a phosphate transporter in a plant or plant part or other photosynthetic organism comprising: (a) inducing protein expression by depriving the plant, plant part, or other photosynthetic organism of sufficient levels of available phosphorus; (b) contacting a portion of a plant, plant part, or other photosynthetic organism with antibody to the protein so that an antibody:antigen complex is formed by the binding of the antibody to an epitope of the protein; and (c) detecting the antibody:antigen complex;

wherein the detection of the antibody:antigen complex is indicative of the expression of the protein.

Transgenic Plants and Photosynthetic Organisms

The classical approach to optimizing plant characteristics utilizes traditional plant breeding methods wherein plants with desirable traits are crossed to produce new, true-breeding cultivars carrying the traits. This approach has at least two serious drawbacks. First, traditional plant breeding relies on the availability of desired traits within known cultivars. The desired phosphate-related traits discussed *supra* are either not part of the genome of certain species or varieties or cannot be acquired to the required level of expression in plants wherein they do occur. Second, traditional breeding is a relatively slow process.

A more modern approach to modifying the characteristics of plants (and other photosynthetic organisms) is to subject plants to mutagenesis by radiation or chemical treatment. Such exposure randomly generates mutations in the DNA molecules comprising the plant genome which sometimes produces the desired traits. The mutagenized plants are screened for the traits and subsequently bred. While mutagenesis has the advantage of producing variations in plant DNA much faster than natural selection, it is not possible to select and generate preferred traits; the process is random. Further, exposing plants to mutagenic agents can induce additional, undesirable mutations to the plant genome. Some of these may not be immediately apparent and, further, may not be able to be "bred out" of a plant carrying a useful mutation.

The nucleic acids and vectors of the invention can also be used to produce transgenic plants and other photosynthetic organisms which express the protein of the invention. The genome of a transgenic organism includes an integrated DNA transgene that was introduced either into that particular organism or into its ancestor. The introduced DNA including the transgene can comprise regulatory element(s) appropriate for the type of organism or tissue being transfected. For example, when introduced as a transgene, a nucleic acid of the invention can be operatively linked to a tissue-specific DNA regulatory sequence such that protein kinase is specifically produced in the target tissue. A seed-specific promoter would permit expression of the protein kinase only in seeds. Also, a suitable 3' region such as the 3' region containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes can be included. Or, other suitable 3' sequences derived from any characterized gene from plants as well as from other organisms such as animals, if they are deemed appropriately functional in the environment of a transgenic plant cell, can be used. To aid in identification of transformed plant cells, the constructs of this invention can be further manipulated to include genes coding for plant selectable markers.

Thus, genetic engineering provides a method of producing a transgenic plant or other photosynthetic organism having altered growth, reproduction, or metabolic content by introducing into a cell or tissue of a plant or other photosynthetic organism, an exogenous nucleic acid which encodes a *psr* protein, such as a β-glucosidase, protein kinase or phosphate transporter, which is transcribed under conditions of phosphate

deprivation in naturally-occurring species in which it occurs, and whose presence in the transgenic plant or photosynthetic organism results in altered growth, reproduction, or metabolic content, and by maintaining the cell or tissue containing the exogenous nucleic acid under conditions appropriate for growth of the cell or tissue, whereby a transgenic plant or other photosynthetic organism having an altered growth, reproduction, or metabolic content can be produced. More specifically, the nucleic acids comprising the *psr*PK, *psr*3.2, *psr*3.1A, *psr*3.1B genes and homologues can be used in this method to produce a transgenic plant from a species which belongs to the vascular plants including angiosperms, gymnosperms, monocots, and dicots, the nonvascular plants, and the algae. Thus plants and other photosynthetic organisms are provided wherein the naturally-occurring phosphate-starvation induced protein kinase or β-glucosidase activity is increased. Because the introduced gene is stably integrated into the genome, seed of a transgenic plant and, therefore future generations of descendants, with this alteration, are also provided by this invention.

Applications and Utilities

Photosynthetic organisms have evolved a number of adaptive strategies to cope with growth-limiting amounts of exogenous inorganic phosphate. These strategies include enhancing the availability of endogenous phosphate (Lefebvre *et al.*, 1990; Sachay *et al.*, 1991), and using it efficiently in order to maintain essential metabolic pathways (Duff *et al.*, 1994), as well as, in times of plenty, storing excess phosphate in vacuoles (Lee *et al.*, 1990; Mimura *et al.*, 1990; Tu *et al.*, 1990)

so that it can later be used to replenish the cytoplasmic pool as required (Rebeille *et al.*, 1983). Also, root systems secrete acids and phosphatases that increase phosphate availability by releasing P_i from rock phosphate and phosphate esters, respectively (Lefebvre *et al.*, 1990; Sachay *et al.*, 1991). The mechanisms of these relief strategies involve changes in either protein synthesis and degradation (Duff *et al.*, 1991), or secretion of pre-existing proteins, including phosphatases (Goldstein, 1992). They invoke changes in phosphate-dependent reactions of photosynthesis (Rao *et al.*, 1990; Usuda and Shimogawara, 1993), respiration (Duff *et al.*, 1989b; Duff *et al.*, 1994; Hoefnagel *et al.*, 1993; Nagano and Ashihara, 1993; Rychter and Mikulska, 1990; Theodorou and Plaxton, 1993), nucleotide synthesis (Ashihara *et al.*, 1988; Rychter *et al.*, 1992), protein synthesis (Sadka *et al.*,1994) and synthesis of cell wall and other metabolites (Fife *et al.*, 1990).

The physiological consequences of P_i limitation in the *Brassica nigra* (*B. nigra*) cell culture system have been studied extensively. These include the accumulation of lipids, starch and phenolic compounds (Fife *et al.*, 1990), an elevated potential for P_i absorption (Lefebvre *et al.*, 1990) and the apparent deployment of alternative enzymes that act to "bypass" P_i- or nucleotide-dependent reactions (Duff *et al.*, 1989b; Duff *et al.*, 1994; Theodorou and Plaxton, 1993).

Recently, in an effort to define components of the regulation of homeostasis of P_i concentration in plant cells, a mutant of *Arabidopsis thaliana*, *pho2*, was isolated that accumulates excess P_i in the shoot (Delhaize and Randall, 1995). The authors suggested that this mutation could affect the regulation of P_i transport

across cell membranes. Another mutant, *pho1*, of this species identified previously is defective in loading of P_i into xylem (Poirier *et al.*, 1991).

It has been proposed that ribonucleases and phosphatases act in tandem to cleave and dephosphorylate RNA molecules in a P_i recycling process (Goldstein, 1992). One extracellular (Glund and Goldstein, 1993; Nurnberger *et al.*, 1990) and four intracellular ribonucleases were reported to be induced in P_i-starved tomato (Loffler *et al.*, 1992). The activities of phosphatase enzymes are known to increase in plants experiencing P_i deficiency (Duff *et al.*, 1989a; Duff *et al.*, 1991; Duff *et al.*, 1994; Goldstein *et al.*, 1989) and it has been shown that acid phosphatases are synthesized *de novo* in P_i-starved cell cultures of *B. nigra* (Duff *et al.*, 1989b). The synthesis of the α-subunit of PP_i-dependent phosphofructokinase is also induced in *B. nigra* cells under P_i stress (Theodorou *et al.*, 1992). Recently, high concentrations of P_i have been shown to down-regulate a sucrose-inducible phosphatase gene (*vspB*) in soybean (Sadka *et al.*, 1994).

A P_i-starvation inducible β-glucosidase could be involved in the deglycosylation and, hence regulation of, certain enzymes during P_i stress (Ballou and Fisher, 1986; Gellatly *et al.*, 1994).

The nucleic acids, constructs, and methods of this invention can be applied to regulate aspects of the phosphate-starvation response of photosynthetic organisms, as well as phosphate metabolism of such organisms in general. By altering the activity of a *psr* protein, *i.e.*, changing the level of expression or modifying its activity, a change in regulation of the phosphate metabolism pathways can be effected. Thus,

phosphate metabolism efficiency could be improved, allowing growth of organisms in phosphate-poor soils without addition of phosphate fertilizers, or improved growth even in phosphate-sufficient environments. Reducing the amount of phosphate fertilizer required for crop plants and/or improvement in yields, would have significant and desirable economic and environmental ramifications.

Also, the timing of certain plant phenomena that depend on phosphate metabolism can be altered. For example, the temporal and quantitative aspects of flowering can be modified in photosynthetic organisms in which reproductive evocation is responsive to the ratio of nitrogen to phosphorus in their environments. Earlier flowering would shorten the growing season for crops and reduce the seed-to-flowering time for bedding plants. Conversely, delay in flowering is desirable in crops harvested for their biomass, such as lettuce and spinach.

Alteration of phosphate metabolism of plants can also result in an altered biomass ratio between root and shoot, and be used to produce a commercial benefit in root crops, such as carrots. Additionally, the ability to induce larger root systems on plants early in the growing season would contribute to drought tolerance later during drier months. Further, if a plant is used to produce a protein in quantity for subsequent purification, it can be differentially expressed in the roots of the plant, and the larger roots in plants of this invention can be especially useful to increase the root biomass and the resulting yield of harvested protein.

Altering cellular Pi levels can also affect the response of a photosynthetic organism to cold and/or frost.

Plants in the field show improved growth in the cold if the plants are phosphate limited. Further, inorganic pyrophosphatase has been shown to increase sucrose levels in the leaves of plants, which may, in part by decreasing Pi levels at the same time, improve cold acclimation and frost tolerance. Thus, one method of increasing cold and frost tolerance in photosynthetic organisms could comprise inhibition or other alteration of *psr* protein expression.

Another embodiment of this invention is the increase in expression of a phosphate transporter protein, such as *psr6* or a functional portion thereof in a photosynthetic organism to increase absorption of phosphate from the environment. These proteins can also be used in phytoremediation applications. For example, Pi deficiency can be stimulated with regulatory protein genes which make the plant or other photosynthetic organism absorb and store more Pi because of the inefficiency of use of Pi.

Further, photosynthetic organisms can be modified to increase the nutritive value of vegetative or reproductive organs. For example, seed plants such as canola, soybean and corn, store phosphate in the form of phytate (the salt of 1, 2, 3, 4, 5, 6-cyclohexanehexolphosphoric acid). The presence of phytate is a problem where the seed is made into meal and used as feed for animals. Monogastric animals cannot metabolize phytate and utilize its phosphate. In addition, phytate binds to essential minerals, such as calcium, manganese and zinc, making them relatively

unavailable to the animal. Alteration of phosphate metabolic pathways in plants could be altered to reduce the level of phytate in seeds in favour of a phosphate storage form that is more usable for animals and humans.

The advantages of the compounds and methods of this invention are multifold and those of skill in the art will recognize that the examples given above are just a few of the applications provided by this invention.

Increased Expression of Protein Kinase

The following examples indicate what can occur if *psr*PK is over expressed or modified to increase its activity. Part of this invention is also to implement the opposite strategy in which this gene or its homologues are underexpressed, modified, or "knocked out" to decrease activity. Those of skill in the art will recognize that similar techniques can be used to alter the expression of other *psr* proteins.

General constitutive promoters can be employed to increase expression of *psr*PK and similar genes throughout the transformed organism to increase its ability to utilize phosphate, thereby growing better under conditions of phosphate limitation and imparting the ability of the plant or other organism to grow better under any regime of phosphate nutrition.

Tissue-specific expression in the roots can have the effect of increasing root size as well as increasing the root's ability to acquire phosphate from the environment and to use it more efficiently in their root metabolism.

Shoot-specific expression is anticipated to alter the temporal aspects and magnitude of flowering, as well as increasing the efficiency of phosphate utilization in the shoot.

Seed-specific expression can alter the efficiency of phosphate utilization in the seed thereby causing more seeds to set and larger seeds to be formed. This strategy can also reduce phytate storage pools in the seed. This can also increase the starch and oil storage capacity of the seed. Phosphate-deprived *B. nigra* suspension cells store more fixed carbon than phosphate-sufficient cells (Lefebvre *et al.* 1990).

Other tissue-specific expression sites can include root hairs for increased phosphate uptake, and other tissues where excessive or inadequate expression can be deleterious to cells and can cause cell death. This can be employed in the production of male sterile lines for hybridization purposes, among other applications.

Methods of Reducing Protein Kinase Expression

The invention encompasses methods of reducing expression of a nucleic acid or a protein of the invention. For example, a transgenic organism can be produced which expresses a molecule that binds directly to an endogenous *psr*PK protein and reduces its protein kinase activity or its ability to bind a substrate or cofactor. Further, expression of a molecule which binds to a *cis*-acting regulatory element or a *trans*-acting regulatory factor so as to interfere with their function can decrease protein kinase expression.

A third method can employ the modification of a nucleic acid or a protein such that it is dysfunctional and interferes with the native (naturally-occurring) functional protein in any way so that its protein kinase activity is reduced or eliminated.

A fourth method can employ an antisense molecule as described in section II above. The inventors contemplate

that, when an antisense molecule of the invention is delivered to target plant cells, it will hydrogen bond with endogenous nucleic acid molecules encoding the protein kinase, thereby reducing gene expression of the protein kinase. The antisense molecule can be designed such that its region of complementarity with an endogenous protein kinase-encoding nucleic acid molecule includes the initiation codon of the sense strand. The antisense molecule can include regions complementary to coding or noncoding regions of the sense strand, or both.

A fifth method can employ transformation such that the introduced nucleic acid comprising part or all of the nucleic acid of the invention in the sense orientation reduces protein kinases expression by any means including gene co-suppression (Meyer and Saedler, 1996).

Although this invention is described in detail with reference to preferred embodiments thereof, these embodiments are offered to illustrate but not to limit the invention. It is possible to make other embodiments that employ the principles of the invention and that fall within its spirit and scope as defined by the claims appended hereto.

All references cited herein are hereby incorporated by reference.

EXAMPLES

Example 1: Plant cell culture

Non-photosynthetic, rapidly growing *Brassica nigra* cell suspensions were cultured as described (Lefebvre *et al.*, 1990) in MS medium (Murashige and Skoog, 1962) containing 6% sucrose (17.5 mM), and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) at 24°C with 130

r.p.m. rotary shaking. To assure the same nutritional state for all cultured cells, 6 ml of 7-day old cultures containing 3 ml of cells (packed cell volume) were inoculated into 44 ml of fresh MS medium containing 1.25 mM P_i. Packed cell volume was determined by allowing the cells to settle from the culture medium in sterile graduated cylinders for 45 min. After 7 days of culture, the same quantity of cells was subcultured into 44 ml of fresh MS medium containing either 10 mM, 1.25 mM, or no phosphate and incubated for an additional 7 days. For the 10 mM P_i treatments, filter-sterilized KH_2PO_4 adjusted to pH 5.8 with KOH was added to the 1.25 mM P_i medium. In the minus P_i treatments, an equal molarity of KCl replaced the KH_2PO_4.

The patterns of growth and endogenous phosphate concentrations of these cultured cells are shown in Figure 1. Cells fed with 10 mM P_i had ample supplies of this nutrient for the duration of the culture period. Cells fed with 1.25 mM P_i, in comparison to minus P_i-treated cells, underwent only mild P_i deprivation by relying on conversion of their own internal phosphate. Quantitative determination of endogenous phosphate was performed as previously described (Lefebvre *et al.*, 1990).

After 7 days growth, the cultured cells were collected on fritted glass filters with 10 μm pore size, and rinsed with 100 ml of 0.5 mM $CaCl_2$ before freezing in liquid nitrogen and storage at -70°C until required.

Example 2: Extraction of total RNA and mRNA

Total RNA from the harvested *B. nigra* cells of Example 1 was isolated as described by Chirgwin (Chirgwin *et al.*, 1979). The cells were homogenized at ice-cold

temperature using a homogenization buffer containing 4 M guanidinium isothiocyanate (GIBCO/BRL, Burlington, Canada). Poly(A)$^+$ RNA (mRNA) was purified by using the mRNA purification kit and recommended protocol of Pharmacia (Uppsala, Sweden and Piscataway, NJ). Total RNA and mRNA isolated from minus P_i-treated, 1.25 mM P_i-fed and 10 mM P_i-fed cells were quantified spectrophotometrically and examined on formaldehyde/agarose gels (Sambrook *et al.*, 1989).

Example 3: Analysis of changes in the populations of mRNA species using *in vitro* translation

It is well known that the protein synthesis profiles of P_i-starved and P_i-fed *B. nigra* cells differ. Such differences in response to P_i starvation could be mediated in various ways. To investigate whether any alterations in protein levels are due to changes at the transcriptional level, *in vitro* translations of mRNA extracted from P_i-starved and P_i-fed *B. nigra* cells were compared. *In vitro* translation of mRNA purified as above was carried out according to the protocol of Promega (Madison, WI), using wheat germ extract (Promega) and [^{35}S]-methionine (ICN, Costa Mesa, CA) diluted with unlabeled methionine to a final concentration of 0.5 mCi/ml.

After translation, a 5 μl aliquot was removed from each reaction mixture and combined with 20 μl of gel-loading buffer to a final concentration of 1% SDS (Laemmli, 1970). The samples were placed in a boiling water bath for 5 min and then subjected to denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using an LKB 2010 Macrophor electrophoresis apparatus (Pharmacia Biotech, Inc., Baie d'Urfé, Canada) and the

discontinuous system of Laemmli (Laemmli, 1970). Molecular weight standards electrophoresed in parallel were ^{14}C-labelled α-lactalbumin, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, chicken egg albumin and bovine serum albumin having molecular weights of 14, 29, 36, 45 and 66 kDa, respectively (Sigma, St. Louis, MO). The gels were 0.4 mm thick slabs containing 1% SDS. The acrylamide monomer concentrations were 5% (w/v) for the stacking gel and 10% for the separating gel. The separating gel was 35 cm long, to maximize resolution of protein species. Electrophoresis was performed at 25°C for 5 h at a constant current of 30 mA.

Upon completion of electrophoresis, the gel, bound to a bind-silane-treated (Pharmacia) glass plate, was incubated in 600 ml of fixing solution containing 20% trichloroacetic acid (TCA) and 10% methanol for 20 min. The gel was then washed three times in 600 ml of post-fixing wash solution containing 10% ethanol and 5% acetic acid, and dried to the glass plate overnight in a fume hood at room temperature. The dried gel was exposed to X-ray film using a Cronex intensifying screen (DuPont, Wilmington, DE) at -70°C and the film was developed using Kodak developer and fixer solutions. The autoradiogram was inspected visually, and scanned using an LKB enhanced UltraScan XL Laser Densitometer (Pharmacia). The densitometric scan was analyzed with GelScan XL Software, Version 2 (Pharmacia).

Results of such analysis are presented in Figure 2. It was assumed for convenience that the density of each band was proportional to the amount of that polypeptide synthesized *in vitro* and, therefore, also proportional to the amount of mRNA coding for the polypeptide. Differences in the densities of corresponding bands

between different P_i treatments were thus taken to represent differences in the abundance of the mRNA species that produced that band. One should nonetheless keep in mind that, since the proteins were labelled with ^{35}S-methionine, the signal is proportional to the methionine content of each polypeptide; to compensate somewhat for this factor, only signals of the same molecular weight were compared between treatments.

The majority of [^{35}S]-methionine-labelled translation products were detected in all P_i treatments. Sixteen polypeptides, however, varied in their signal intensities among the three treatments. Figure 3 is a summary of the standardized data of the expression differences between polypeptides produced from 10 mM P_i-fed, 1.25 mM P_i-fed and P_i-deprived cells. Based on these analyses, P_i deprivation caused the copy number of mRNAs to increase for ten polypeptides, whereas six others decreased. Of translatable mRNAs showing altered expression, four species corresponding to proteins with estimated molecular weights of 31.7, 32.3, 52.5, and 64.8 kDa were only detected in the P_i-starved treatment. By comparison with the other polypeptides, these were expressed at relatively high levels during P_i deprivation. The repression of a 43.5 kDa polypeptide was also noted in minus P_i-treated cells. The other six polypeptides representing mRNAs preferentially expressed during P_i starvation had estimated molecular weights of 19.6, 40.6, 30.1, 37.0, 18.6, and 29.5 kDa. These were expressed at approximately 3.3, 2.8, 2.7, 1.7, 1.3, and 1.2 times higher levels, respectively, in the minus P_i-treated cells than in the 10 mM P_i-fed cells. In addition, there were 20-, 6.6-, 1.8-, 1.6-, and 1.4-fold decreases in the minus P_i-treated cells in the expression of polypeptides

with estimated molecular weights of 14.4, 35.9, 48.3, 18.4, and 35.5 kDa, respectively. In the 1.25 mM P_i-fed cells, the mRNAs coding for the sixteen differentially expressed polypeptides appeared to be present either at intermediate levels or at levels similar to those in the minus P_i-treated cells.

No difference was detected between the patterns of gel electrophoresis of the *in vitro* translation products when using either total RNA or poly(A)$^+$ RNA. The above results were consistent in three independent experiments.

Example 4: Construction of cDNA library from minus P_i-treated *Brassica nigra* cells

Approximately 10 μg of poly(A)$^+$ RNA purified from cells deprived of P_i for 7 days was used to synthesize a double-stranded cDNA library using the c-CLONE II cDNA Synthesis kit (Clontech, Palo Alto, CA) according to manufacturer's instructions. The oligo-d(T)-tailed cDNA products produced were fractionated on a Chroma Spin-400 column (Bio/Can Scientific, Mississauga, Canada) to obtain cDNA molecules greater than 400 bp in length. The size range of such molecules was estimated to be from about 0.5 kb to about 3 kb in length. Using *Eco*RI restriction site sticky ends, the size-selected cDNA molecules were ligated into *Eco*RI predigested/phosphatase-treated λZAPII bacteriophage arms as recommended by the manufacturer (Stratagene, La Jolla, CA). Recombinant phages were packaged with Stratagene's Gigapack II Gold and titered on a lawn of *E. coli* XL1-Blue bacteria grown on LB agar plates according to the manufacturer's protocol. The ligation efficiency at an optimal ratio of cDNA: vector (44 ng: 500 ng) was 7.5 x 10^6 plaque forming units (pfu)/μg cDNA.

Example 5: Differential hybridization and cross-hybridization screening of the cDNA library from minus P_i-treated *Brassica nigra* cells

The cDNA library from minus P_i-treated *B. nigra* cells described above was differentially screened by hybridizing to cDNA probes from both minus P_i-treated and 10 mM P_i-fed *B. nigra* cells, in order to identify genes responsible for the differences in protein and mRNA profiles between these cells.

Single-stranded cDNA probes were synthesized from mRNAs isolated from the P_i-deprived and 10 mM P_i-fed cells to make -P and +P probes, respectively. This was done as previously described (Sambrook *et al.*, 1989), using a mixture of 5 μg of mRNAs and 7.4 μg of random hexadeoxyribonucleotides (Pharmacia). Incorporation of radiolabel was achieved by including α-^{32}P-dATP (ICN) in the reaction mixture. Reaction products were extracted with phenol:chloroform and purified on Sephacryl S-300 columns (Pharmacia).

The unamplified cDNA library was plated on a lawn of *E. coli* strain XL1-Blue growing at low density (2,000 pfu per 90 mm LB plate). A total of about 50,000 plaques were screened as follows: First, duplicate lifts were prepared from each plate. The plaques' DNA was denatured and immobilized on nitrocellulose filters (Amersham, Oakville, ON, Canada) by standard methods (Sambrook *et al.*, 1989). The filters were baked, prewashed in a solution of 5X SSC, 0.5% SDS, 1 mM EDTA (pH 8.0) for 1 h at 68°C and prehybridized in a solution of 6X SSC, 0.05X BLOTTO, 25 μg/ml of denatured, fragmented salmon sperm DNA (Pharmacia) at 68°C for 2-3 h. (1X BLOTTO contains 5% non-fat dried milk (Carnation Inc., Toronto) and 0.02% sodium azide (Sigma).) For each pair of duplicate

filters, one was hybridized to the radiolabelled -P probes and the other to the radiolabelled +P probes at 68°C overnight. The filters were then washed under conditions of increasing stringency as follows: three times in 2X SSC, 0.1% SDS at room temperature for 5 min.; twice in 1X SSC, 0.1% SDS at 68°C for 1-1.5 h; and once in 0.2X SSC, 0.1% SDS at 68°C for 1 h. (1X SSC solution consists of 0.15 M NaCl and 15 mM trisodium citrate.) Autoradiography was performed as described above. Clones that hybridized to -P probes but not to +P probes were identified as "positive". These clones were then subjected to second and third rounds of screening.

Three rounds of screening resulted in the isolation of 131 clones that were preferentially expressed in the starved cells. Because the total number of plaques screened was deliberately large (so as not to miss anything), the 131 clones included multiple separate cloning events of the same gene(s). Therefore, it was necessary to cross-hybridize the isolated clones to each other, in order to distinguish duplicate from novel cloning events.

The isolated clones were digested with *Eco*RI, *Pst*I and *Taq*I restriction enzymes and the resulting DNA fragments were separated on 1% agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) in 10X SSC overnight (Sambrook *et al.*, 1989). ^{32}P-labelled single-stranded probes were generated from the liberated *B. nigra* inserts of individual clones using PCR (Konat *et al.*, 1991), employing T_3 and T_7 primers in the first round and either T_3 or T_7 primers in the second round of reactions.

Membranes to which DNA from the isolated clones was bound were first baked for 30 min. at 80°C, then

prehybridized for 5 min. at 65°C in 0.25 M NaH_2PO_4 (pH 7.2), 7% SDS, 1 mM EDTA. Next, radiolabelled probe was added and hybridization was allowed to proceed for 2 hr. The membranes were then washed twice in 40 mM NaH_2PO_4 (pH 7.2), 5% SDS, 1 mM EDTA and twice in 40 mM NaH_2PO_4 (pH 7.2), 1% SDS, 1 mM EDTA, each time for 30-60 min. at 65°C. Autoradiography was as described above. The membranes were stripped of bound probe by washing twice in 0.1X SSC and 0.5% SDS at 95°C for 20 min.

The results of the cross-hybridization analysis (not shown) permitted the majority of the P_i-inducible clones to be placed into eleven different homology groups, which were designated plant "P_i-starvation responsive" (*psr*) groups 1 to 11. Because these genes are particularly active during periods where phosphate is unavailable, they are assumed to be involved in the plant response to phosphate starvation.

Example 6: RNA analysis of psr cDNA clones using northern blots

The induction and relative abundance of *psr* mRNAs under the different phosphate growth conditions were further assessed by northern blotting, results of which are shown in Figure 4.

Cloned *psr* cDNA inserts were liberated from vector DNA by digestion with *Eco*RI. The reaction products were electrophoresed on a 1% agarose gel and bands containing insert DNA were excised. This DNA was subsequently purified using GeneClean II (Bio 101 Inc., Vista, CA) and radiolabelled by random-priming reactions (Sambrook *et al.*, 1989). The radiolabelled probes were purified using Chroma Spin-30 columns (Clontech, Palo Alto, CA). A

control DNA probe (α-tubulin) was similarly radiolabelled and purified.

30μg of total RNA extracted from each of the minus P_i-treated, 1.25 mM P_i-fed and 10 mM P_i-fed cells were electrophoresed on a 2.2 M formaldehyde/ 1% agarose gel (Sambrook *et al.*, 1989) and transferred to Nytran Plus membrane (Schleicher & Schuell) according to the manufacturer's protocol.

Individual blots were incubated at 42°C for 30 min. in prehybridization buffer that contained 50% formamide, 0.12 M NaH_2PO_4 (pH 6.8), 0.25 M NaCl, 7% SDS, and 1 mM EDTA. They were transferred to 10 ml of fresh hybridization buffer containing 2-5 x 10^7 cpm of probe at approximately 4 x 10^9 cpm/ μg specific activity. Hybridization was overnight at 42°C followed by washing for 30 min. each in 2X SSC, 0.1% SDS at room temperature; 0.5X SSC, 0.1% SDS at room temperature and 0.1X SSC, 0.1% SDS at 65°C. Autoradiography was for 1-4 days. To reuse the blots, bound probes were removed by washing in 0.1% SDS for 5 min, followed by equilibration in 5X SSC, 0.1% SDS at room temperature for 20 min. and stripping in the same solution for 2 min. at 95°C.

Based on comparisons to the α-tubulin internal standard, most of the *psr* genes were relatively highly expressed and all were induced in both the minus P_i-treated and the 1.25 mM P_i-fed cells, with somewhat less expression in the latter cells. In 10 mM P_i-fed cells, low levels of mRNA expression were observed for *psr7, 8, 9, 10 and 11*, whereas transcripts for the other genes were not detected. High stringency washing did not remove the additional bands seen in all P_i treatments when probing with *psr9, 10* and *11*. For each of these,

the differentially expressed mRNA species was the smallest size of those detected.

Example 7: Nuclear run-off experiments

Plasmid DNA carrying the *B. nigra psr1* cDNA or *A. thaliana* α-tubulin inserts was alkaline denatured and applied to Nytran-Plus membranes (Schleicher and Schuell, Guelph, Canada) using Bio-Dot Microfiltration apparatus (Bio-Rad) at five μg per dot. In order to produce probe from newly synthesized mRNA species, transcriptionally active nuclei were isolated from 5-6 g of 5 mM P_i-fed and P_i-starved root tissues as described (Willimizer and Wagner, 1981). Transcription was allowed in the presence of ^{32}P-UTP for 60 min at 30°C using the method of Chappel and Hahlbrock (1984). Labelled RNAs were purified as described by Somssich *et al.* (1989). Approximately 10^6 cpm of RNA probe was used for hybridization with the dot blots as described (Malboobi and Lefebvre, 1995). After the last wash, the blot was treated with 20 μg/ml of ribonuclease A in 2X SSC for 30 min at room temperature. The blot was then washed with 2X SSC, 0.5% SDS, twice and 2X SSC, twice. Dot blots were exposed to X-ray film for 7 or more days. The results are shown in Figure 10.

Example 8: Plant culture

Arabidopsis thaliana (var. Columbia) seeds were surface sterilized for ten minutes in 30% bleach (Javex), 0.03% Triton X-100 (Sigma) and washed with sterilized water six times. Seeds were transferred onto a 1 cm^2 piece of steel mesh placed on solid MS (Murashige and Skooge) plates containing 0.5% agar and 2% sucrose. After 11 days, plantlets grown and rooted through the

mesh were transferred to 15 ml of half strength liquid MS medium containing 1.25 mM P_i and 1% sucrose in 125 ml Erlenmeyer flasks at 24°C, 540 lux light with 80 r.p.m. rotary shaking. Three days later, plants were transferred into MS medium with various concentrations of nutrients as follows. For treatments with differing concentrations of Pi, filter-sterilized KH_2PO_4 adjusted to pH 5.8 with KOH was added to the 1.25 mM P_i medium up to 5 mM. For the minus P_i treatments, filter-sterilized KCl replaced the KH_2PO_4 to 5 mM concentration.

Example 9: DNA sequencing and computer analysis

The *psr* cDNA inserts were excised *in vivo* from selected recombinant λZAPII phages into Bluescript™ plasmid vectors in the presence of R408 helper phage according to supplier's instructions (Stratagene). Plasmid DNA was prepared by CsCl gradient centrifugation (Sambrook *et al.*, 1989).

Both strands of the isolated DNA was then sequenced using a Sequenase Version 2 Kit (United States Biochemical Corp., Cleveland, OH) with T_3 and T_7 primers, and other appropriate primers along the sequence (Stratagene). Sequencing of the 5' and 3' ends of these clones immediately indicated clear distinctions between the eleven *psr* groups.

Homology searches on the obtained sequences were performed with the BLASTX program (Gish *et al.*, 1993) against the GenBank database through NCBI (The National Center for Biotechnology Information). Assembling and editing of the coding sequence and analysis of DNA and predicted protein sequences were performed using related programs of LASERGENE software for Macintosh (DNASTAR, Madison, WI).

Clone *psr1* was identified as a gene coding for a phosphate-starvation inducible protein kinase. It has therefore been given the additional name *psr*PK. The *psr*PK sequence possesses high homology to protein kinases isolated from other plants, such as *Glycine max* protein kinase 2 (SPK2, GB accession #L19360, 63% homology), *Arabidopsis thaliana* protein kinase 2 (ASK2, GB Accession # Z12120, 70% homology) (Park *et al.*, 1993), *Brassica napus* serine threonine kinase 1 (BSK1, GB Accession #L12393, 69% homology), *Glycine max* protein kinase 3 (SPK3, GB Accession #L19361, 76% homology), *Brassica napus* serine threonine kinase Z (BSKZ GB Accession # L12394, 71% homology), *Arabidopsis thaliana* protein kinase 1 (ASK1, GB Accession # P43291, 71% homology) (Park *et al.*, 1993).

An *A. thaliana* λPRL2 cDNA library (CD4-7) was obtained from the Ohio State *Arabidopsis* Biological Resource Center. It was screened at high stringency with a probe consisting of the entire *B. nigra psr*PK cDNA insert. The *B. nigra psr*PK cDNA insert was used for random priming probe synthesis and subsequent hybridization in non-radioactive Du Pont Renaissance™ kit according to the manufacturer's instructions (Du Pont NEN, Boston, MA). A strongly hybridizing plaque (designated *psr1-1*) was purified and its bacteriophage DNA isolated. The *A. thaliana* insert of the phage was subjected to dideoxy nucleotide sequencing of both strands after *in vivo* excision from the Bluescript plasmid.

Figure 5 shows the cDNA sequence of *B. nigra psr*PK, aligned with the cDNA sequence of the corresponding protein kinase from *Arabidopsis thaliana*.

Example 10: Genomic clone isolation

A genomic library of *A. thaliana (Var. Colombia)* in EMBL3 was screened at high stringency with a probe consisting of the unique 3' sequence of the *A. thaliana* *psr*PK cDNA insert. This insert was used for random priming probe synthesis and subsequent hybridization in non-radioactive Du Pont Renaissance™ kit according to the manufacturer's instructions (DuPont NEN, Boston). Positive clones were then rescreened until homogenous and subjected to Southern blot analysis using the same probe as above. Inserts from positive clones were excised and digested with appropriate restriction enzymes and subcloned into plasmids. The inserts were then sequenced by dideoxy nucleotide sequencing of both strands.

Example 11: Plant transformation with *psr*PK constructs

Arabidopsis thaliana was transformed *in planta* with eight different constructs employing constitutive and tissue-specific promoters attached to sense and antisense nucleic acids of the entire and partial sequences of *psr*PK from *A. thaliana*. See Figures 12A-12D, **Table 2 and Figure 28.**

In some constructs (C1, C7, D1, D4, PA1, PA3 and AA5, see Table 2), the GUS gene present in the pBI121 vector (CLONTECH, Palo Alto, CA, www.clontech.com) was replaced by the *psr*PK gene in either a sense (*psr*1) (Figure 12A) or antisense (α-*psr*1) (Figure 12B) direction to produce a sense or antisense construct under the control of a constitutive (CaMV-35S) promoter. The CaMV-35S promoter (cauliflower mosaic virus 35S promoter) of pBI121 is fused upstream of GUS gene. The GUS gene was removed from this vector by *Sma*I and *Eco*ICRI digestion. A cDNA encoding *psr*PK was cloned in pZL1 vector (isolated

from *Arabidopsis* Resource Center cDNA library). This clone was digested by *Sma*I and *Bam*HI enzymes. The *Bam*HI site was subsequently filled in by Klenow fragment of DNA polymerase I. The resultant vector and *psr*PK gene were subjected to blunt-end ligation. As a result, both sense and antisense construct of *psr*PK genes under the control of CaMV 35S promoter were obtained. In other constructs (Nos. 2, 6 and 26, psr1, No. 2 (P1+P2), No. 12 (P3+P4)), *psr*PK was directionally cloned in the *Bam*HI/*Sma*I sites of pBI121 between promoter and GUS gene without removing the latter.

Competent *E. coli* DH 5-α was transformed by the ligation products and plated on LB/kan. The growing colonies were picked and mini-prepped. Digestion with *Sal*I and *Eco*RI enzymes distinguished the sense and antisense constructs by appearance of 1.6 Kb and 0.3 Kb fragments, respectively.

Constructs depicted in Figures 12C and 12D were produced in a similar manner except that the promoterless pBI101 vector was used and an Arabin promoter (Arabin-pro) was inserted as a *Hind*III-*Sal*I fragment fused upstream of the GUS gene in the pBI101 vector. The GUS gene present in the promoterless pBI101 vector (CLONTECH, Palo Alto, CA, www.clontech.com) was then replaced by the *psr*PK gene in either a sense (*psr*1) (Figure 12C) or antisense (α-*psr*1) (Figure 12D) direction to produce a sense or antisense construct under the control of a seed-specific (Arabin) promoter.

The *in planta* transformation protocol used is as described by Katavic *et al.* (1994). Briefly, *Agrobacterium tumefaciens* strain GV3101 bearing the helper nopaline plasmid MP90 and a binary vector containing the *psr*PK gene construct and a plant

selectable marker was grown overnight. Wound sites of excised primary and secondary inflorescence shoots were exposed to cultures of the transformed *Agrobacterium* cells three times to inoculate the plant tissues.

The treated plants were grown to maturity, and the seeds harvested and screened for transformants on selective medium (**Figure 28**). Confirmation of transformation was made by determining if the plants contain the transferred genes through Southern blots or polymerase chain reaction techniques using the *psr*PK and associated sequences.

Example 12: Bacterial expression of *psr*PK and determination of protein kinase activity

The *psr*PK protein was expressed in the *E. coli* expression vector pGEX (Promega). The insert was amplified out of the Bluescript plasmid containing the *Arabidopsis psr*PK such that a fragment was produced which contained the translational start and which stopped one codon short of the stop codon (i.e. excluding the stop codon). This fragment was cloned into a modified pGEX plasmid containing six extra codons for histidine at the 3' end of the inserted *psr*PK cDNA. The protein was then expressed in the bacteria and the *psr*PK protein was purified from crude extract using columns which exploit the affinity properties of the added histidine residues.

Protein kinase activity was determined by three different experiments using the purified *psr*PK protein. Manser, *et al.* (1994); Manser, *et al.*, (1992); Manser, *et al.*, (1995).

The first two experiments involve activity determinations on proteins obtained from plants grown minus phosphate. Between 1-5 g of plant tissue was

homogenized in a buffer containing 15 mM Hepes/KOH pH7.6, 40 mM KCl, 5mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethysulfonyl fluoride (PMSF) (Sigma). The homogenate was kept on ice for 30 min. before centrifuging at 13000 xg for 15 min, twice. The protein concentration was determined by using Bio-Rad Protein Assay Reagent Concentrate as described by the manufacturer (Bio-Rad Lab., Richmond, CA). Fifty μg of each protein extract was loaded onto a denaturing SDS-PAGE or a native gel (Laemmli, 1970). For the SDS-PAGE, the final concentration of acrylamide monomer in the 0.75 mm thick mini gel (Bio-Rad) was 4% for the stacking gel and 10% for the separating gel. The SDS-PAGE was run at 200V at room temperature and stained with Coomassie R-250 (Sigma). For the native gel, which lacked SDS, the final concentration of acrylamide monomer was 3% for the stacking gel and 7% for the separating gel. The gel was pre-run at 4°C at 200V for 1 hr prior to loading samples and then run at 200V for 45 min. at 4°C.

The separated proteins were blotted onto Immobilon P PVDF membrane (Millipore, Mississauga, Ontario) and, in the case of the SDS-denatured samples, the blots were exposed to denaturing steps of 6M Guanidine HCl in MES buffer, diluted 50% with MES buffer, five cycles, followed by a renaturing step of PBS buffer for 3 hours. These were then exposed to 25 mM MES, pH 6.5, containing either purified protein and 25 μCi γ^{32}P-ATP or 25 μCi γ^{32}P-ATP alone for 5 min. at 22°C, then 10 min. at 4°C. The blots were then washed, dried, and exposed to X-ray film for detection purposes.

For native gel separation, the blots were immediately exposed to 25 mM MES, pH 6.5, containing either purified protein and 25 μCi γ^{32}P-ATP or 25 μCi

γ^{32}P-ATP alone for 5 min. at 22°C, then 10 min. at 4°C. These blots were also washed, dried and exposed to X-ray film for detection purposes.

The third activity experiment used casein or histones as artificial substrates for *psr*PK (Uesono, *et al.*, (1992)). The *psr*PK protein expressed and purified from bacteria was dialyzed into 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol. An equal volume of this mixture was added to 20 μM ATP, 2 μM/ml dephosphorylated casein (or histone) and 25 μCi γ^{32}P-ATP. The sample was run on an SDS-Page gel as described above and the gel dried and exposed to X-ray film for detection purposes. The *psrPK* protein phosphorylated both casein and histone.

Example 13: Plant culture for *psr* β-glucosidase expression analysis under other stresses

Arabidopsis thaliana (var. Columbia) seeds were germinated and grown essentially as described in Example 8. The concentration of P_i was kept at 5 mM when investigating responses to other environmental stresses. For high salt treatment, sterilized NaCl solution was added to a final concentration of 100 mM, a sublethal concentration (Saleki, *et al.*, (1993). For medium with no nitrogen, KNO_3 and NH_4NO_3 were replaced by an equal molarity of KCl. For medium lacking sulphur, $MgSO_4$ was replaced by an equal molarity of $MgCl_2$. Heat shock was performed by incubating 14 day-old plants at 39°C for 2 hours. Anaerobic conditions were created by blowing argon gas into the flasks containing 13 day-old plants through sterilized tubes plugged with cotton for 24 hours. In all cases, the culture medium was removed and replaced with fresh medium every 4 days to ensure that

there was no depletion of supplied nutrients. Treated plants were harvested after 14 days except for plants grown without nitrogen that were harvested on day 11 due to onset of severe deprivation symptoms, and plants starved for P_i for 14 days and resupplied with 5 mM P_i that were harvested after a further 1 or 3 days. Only P_i deprivation caused significant increases in mRNA levels for β-glucosidase.

Example 14: Southern blots and genomic library screening

Genomic DNA was extracted from *A. thaliana* plant material in a CTAB extraction buffer according to Saghai-Maroof, *et al.* (1984). About 10 μg of genomic DNA was digested with either *Bam*Hl, *Eco*RI, *Sal*l, or *BamH*I/*Sal*I restriction enzymes overnight at 37°C. After separating the digestion products on a 0.8% agarose gel, DNA fragments were transferred to Nytran-Plus membrane as described by the manufacturer (Schleicher and Schuell, Guelph, Canada). The *Brassica nigra psr*3.1 cDNA insert (Malboobi and Lefebvre, (1995)) was used for random priming probe synthesis and subsequent hybridization using the non-radioactive DuPont Renaissance™ system according to the manufacturer (Du Pont NEN, Boston, MA).

A genomic library of *A. thaliana* ecotype Columbia cloned into EMBL3 was provided by Kenton Ko, Queen's University, Kingston, Canada. About 200,000 plaque forming units (pfu) were screened with probe derived from the *B. nigra psr*3.1 cDNA clone under high stringency conditions (Malboobi and Lefebvre, (1995)). Positive clones were carried through secondary and tertiary screening. Probes derived from the isolated genomic DNA inserts were used in Southern blotting to determine which genomic clones corresponded to the *B. nigra psr*3.1. A

genomic clone with a similar blotting pattern to that of *B. nigra psr*3.1 cDNA clone was chosen for restriction enzyme site mapping and subcloning by standard methods (Sambrook, *et al.*, (1989)).

Example 15: RNA isolation and northern blots

Total RNA was extracted from plant tissues according to Chirgwin and colleagues (Chirgwin, *et al.*, (1979)). Twenty five μg of each RNA extract was loaded onto a 1% agarose/formaldehyde gel (Sambrook, *et al.*, 1989). Northern blotting and laser densitometric scanning of autoradiograms were carried out as previously described (Malboobi and Lefebvre, (1995)).

Example 16: Sequence determination and computer analysis

The DNA fragments resulting from *EcoR*I, and *EcoR*I/*Sal*I digestions of the *psr3* genomic clone were prepared and inserted into the pBluescript KS-vector (Stratagene, La Jolla, CA), and these constructs were used to transform competent *E. coli* strain DH5-α (GIBCO BRL, Burlington, Canada) through standard cloning techniques (Sambrook, *et al.*, 1989). Plasmid DNA was prepared with the Wizard™ Megapreps DNA Purification System (Promega, Madison, WI). Both strands of the inserts were sequenced by the dideoxynucleotide method using a Sequenase Version 2 Kit (United States Biochemical Corp., Cleveland, OH). Homology searches of databases were conducted using the BLASTX program (Gish and State (1993)) against DNA and protein sequences. Assembling and editing of the coding sequence and analysis of DNA and predicted protein sequences were performed using the appropriate program of LASERGENE software or Macintosh (DNASTAR, Madison, WI). Similar

sequencing and homology searches were performed for all cDNA clones of *psr* genes.

Example 17: Primer extension analysis

Transcriptional start sites for β-glucosidase were determined by primer extension analysis. The oligonucleotide, AGCAAAAGCGCCCATGAGAGGAA, was labelled with T4 polynucleotide kinase (Promega, Madison, WI) in the presence of [γ-^{32}P]-dATP as described (Sambrook, *et al.*, (1989)). Labelled oligonucleotide was purified using the MERMAID system (Bio/Can Scientific, Mississauga, Canada) and annealed to RNA extracted from P_i-starved roots, then subjected to primer extension by AMV reverse transcriptase (Pharmacia, Baie D'Urfé, Canada) as described in Ausubel, *et al.*, (1995). The resulting products were characterized by standard procedures.

Example 18: Protein extraction and gel electrophoresis for β-glucosidase

Approximately ten mg fresh wt of root tissue were homogenized in 500 μl of a buffer containing 15 mM HEPES/KOH pH7.6, 40 mM KCI, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO). The homogenate was kept on ice for 30 min. before centrifuging at 13000x*g* for 15 min. The supernatant was removed and centrifuged for a further 15 min. The protein concentration of this final supernatant was determined using Bio-Rad Protein Assay Dye Reagent Concentrate as described by the manufacturer (Bio-Rad, Richmond, CA). Fifty μg of total protein were electrophoresed in each lane in either denaturing SDS-PAGE or native PAGE gels

(Laemmli, (1970)). For the SDS-PAGE, the final concentration of acrylamide monomer in the 0.75 mm thick mini-gel (Bio-Rad) was 4% and 10% in the stacking and separating gels, respectively. The gel was run at 200V at room temperature and stained with Coomassie R-250 (Sigma). For the native gel, SDS was eliminated from all reagents and the final concentrations of acrylamide monomer were 3% and 7% for the stacking and separating gels, respectively. The native gel was prerun at 4°C and 100 V for 30 min prior to sample loading and then run at 200V for 3 h at 4°C. To detect β-glucosidase activity, the native gel was equilibrated in a 100 mM sodium acetate, pH 6.5 (Sigma) buffer containing 20 mM $CaCl_2$ for 15 min. This was followed by incubation for 3 hours in the same solution plus 0.02%, (w/v) Fast Garnet GBC salt (Sigma) and 0.04% (w/v) β-naphthyl β-D-glucopyranoside (Sigma) at room temperature.

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Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

DATED this **30th** day of **June 2000**

Queen's University at Kingston

DAVIES COLLISON CAVE

Patent Attorneys for the applicant

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Claims

What is claimed is:

1. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, which nucleic acid comprises:

(a) SEQ ID NO:1, SEQ ID NO:3, or a nucleic acid sequence which encodes YLDANDEE;

(b) a nucleic acid sequence which hybridizes to a nucleic acid sequence which encodes YLDANDEE, in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS; or

(c) a nucleic acid which is complementary to any of the nucleic acid sequences of (a) or (b).

2. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising nucleotide residues 1041 to 1106 of SEQ ID NO:1.

3. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising a nucleic acid sequence which is identical to, which is complementary to, or which hybridizes to nucleotides 1065 to 1088 of SEQ ID NO:1 in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

4. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of

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the nucleic acid is induced by phosphate deficiency in said plant, the isolated nucleic acid encoding an amino acid sequence comprising 40 consecutive amino acids of SEQ ID NO:2 or comprising 20 consecutive amino acids of amino acid residues 318 to 339 of SEQ ID NO:2.

5. The isolated nucleic acid according to claim 1, which nucleic acid is isolated from *Brassica* sp. or *Arabidopsis* sp.

6. The isolated nucleic acid according to claim 1, which nucleic acid is recombinant or synthetic nucleic acid.

7. Isolated DNA encoding a protein kinase of a plant in which transcription of the DNA is induced by phosphate deficiency in said plant, which DNA comprises nucleotides 1065 to 1088 of SEQ ID NO:1.

8. A probe or primer comprising 16 or more nucleotides which hybridizes to nucleotides 1041-1106 of SEQ ID NO:1 in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

9. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising 16 or more contiguous nucleotides of a DNA consisting of nucleotides 1065 to 1088 of SEQ ID NO:1.

10. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising 20 or more contiguous nucleotides of a DNA consisting of nucleotides 1065 to 1088 of SEQ ID NO:1.

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11. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, wherein the nucleic acid hybridizes to a nucleic acid comprising 16 or more contiguous nucleotides of a DNA consisting of nucleotides 1065 to 1088 of SEQ ID NO:1 in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

12. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, wherein the nucleic acid hybridizes to a nucleic acid comprising 20 or more contiguous nucleotides of a DNA consisting of nucleotides 1065 to 1088 of SEQ ID NO:1 in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C with a wash at 42° C in 0.2x SSC and 0.1% SDS.

13. A transgenic cell of a photosynthetic organism containing:
a) the nucleic acid of claim 1;
b) a nucleic acid complementary to the nucleic acid of a); or
c) either (a) or (b), and one or more other exogenous nucleic acids.

14. A cell according to claim 13 which is a root cell.

15. A cell according to claim 13 which is the cell of a seed.

16. A cell according to claim 13 which is a photosynthetic cell.

17. Isolated DNA encoding a protein kinase of a plant in which transcription of the DNA is induced by phosphate deficiency in said plant, which DNA comprises a nucleic acid

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encoding YLDANDEE.

18. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, which nucleic acid hybridizes to a nucleic acid which encodes YLDANDEE in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

19. A probe or primer comprising 16 or more nucleotides which hybridizes to a nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, wherein the probe or primer hybridizes to a nucleic acid encoding YLDANDEE in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0. 1% SDS.

20. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising 16 or more contiguous nucleotides of a nucleic acid which encodes YLDANDEE.

21. An isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, the nucleic acid comprising 20 or more contiguous nucleotides of a nucleic acid which encodes YLDANDEE.

22. Isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, wherein the nucleic acid hydridizes to a nucleic acid comprising 16 or more contiguous nucleotides of a DNA

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which encodes YLDANDEE in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

23. Isolated nucleic acid encoding a protein kinase of a plant in which transcription of the nucleic acid is induced by phosphate deficiency in said plant, wherein the nucleic acid hybridizes to a nucleic acid comprising 20 or more contiguous nucleotides of a DNA which encodes YLDANDEE in a hybridization procedure with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with a wash at 42° C in 0.2x SSC and 0.1% SDS.

24. An isolated polypeptide having protein kinase activity comprising an amino acid sequence which is:
(a) encoded by the nucleic acid of claim 1; or
(b) encoded by nucleotide residues 677 to 1020 of SEQ ID NO:1.

25. An isolated polypeptide having protein kinase activity and an amino acid sequence substantially identical to a sequence selected from SEQ ID NO:2 and SEQ ID NO:4.

26. An isolated polypeptide having protein kinase activity comprising an amino acid sequence which is encoded by the nucleic acid of claim 1, which nucleic acid is isolated from *Brassica* sp. or *Arabidopsis* sp.

27. A recombinant expression vector comprising the nucleic acid of claim 1 operatively linked to a regulatory sequence.

28. An isolated host cell transformed with the vector of claim 27.

29. The cell of claim 28 which is the cell of a photosynthetic organism.

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30. The cell of claim 28 which is a prokaryotic cell.

31. A transgenic plant, plant part, or tissue culture produced using the vector of claim 27 and expressing a protein kinase having an amino acid sequence substantially identical to a sequence selected from SEQ ID NO:2 and SEQ ID NO:4.

32. The plant part of claim 31 which is a seed.

33. The plant part or tissue culture according to claim 31 wherein the plant part or tissue culture is derived from *Arabidopsis* sp. or *Brassica* sp.

34. A method of producing a transgenic plant or other photosynthetic organism having altered growth, reproduction, or metabolism comprising:

(a) introducing into a cell or tissue of a plant or other photosynthetic organism, an exogenous nucleic acid which encodes a protein kinase according to claim 1, said nucleic acid being transcribed under conditions of phosphate deprivation in naturally occurring species in which it occurs, and whose presence in the transgenic plant or photosynthetic organism results in altered growth, reproduction, or metabolic content; and

(b) maintaining the cell or tissue containing the exogenous nucleic acid under conditions appropriate for growth of the cell or tissue,

whereby a transgenic plant or other photosynthetic organism having an altered growth, reproduction, or metabolism relative to a wildtype plant of the same species grown under the same conditions is produced.

35. The method of claim 34 wherein the transgenic plant or other photosynthetic organism is selected from the group consisting of: angiosperms, gymnosperms, monocots, dicots, nonvascular plants, eukaryotic algae, and cyanobacteria.

36. The method of claim 34 wherein the nucleic acid comprises SEQ ID NO:1, SEQ ID NO:3, or a complementary nucleic acid thereof.

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37. The method of claim 34 wherein the naturally occurring phosphate starvation-induced protein kinase activity is reduced.

38. Seed of a transgenic plant produced according to claim 34.

39. An antibody or antibody fragment which binds to sequence YLDANDEE of a polypeptide encoded by the nucleic acid of claim 1.

40. The antibody or antibody fragment of claim 39, wherein the antibody is a polyclonal antibody or a monoclonal antibody, or the antibody fragment is an F*ab* fragment.

41. A method of detecting the expression of a phosphate-starvation inducible protein kinase in a plant or plant part or other photosynthetic organism comprising:

(a) inducing protein kinase expression by depriving the plant, plant part, or other photosynthetic organism of sufficient levels of available phosphorus for growth;

(b) contacting a portion of a plant, plant part, or other photosynthetic organism with the antibody of claim 39 so that an antibody:antigen complex is formed by the binding of the antibody to an epitope of the protein kinase; and

(c) detecting the antibody:antigen complex;

wherein the detection of the antibody:antigen complex is indicative of the expression of the protein kinase.

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42. The isolated nucleic acid according to any one of claims 1-6, or the isolated DNA according to claim 7, or the probe or primer according to claim 8, or the isolated nucleic acid according to any one of claims 9-12, or the transgenic cell according to claim 13, or the cell according to any one of claims 14-16, or the isolated DNA according to claim 17, or the isolated nucleic acid according to claim 18, or the probe or primer according to claim 19, or the isolated nucleic acid according to any one of claims 20-23, or the isolated polypeptide according to any one of claims 24-26, or the recombinant expression vector according to claim 27, or the isolated host cell according to claim 28, or the cell according to claim 29 or claim 30, or the transgenic plant according to claim 31, or the plant part according to claim 32 or claim 33, or the method according to any one of claims 34-37, or the seed according to claim 38, or the antibody according to claim 39 or claim 40, or the method according to claim 41, substantially as herein before described with reference to the figures and/or examples.

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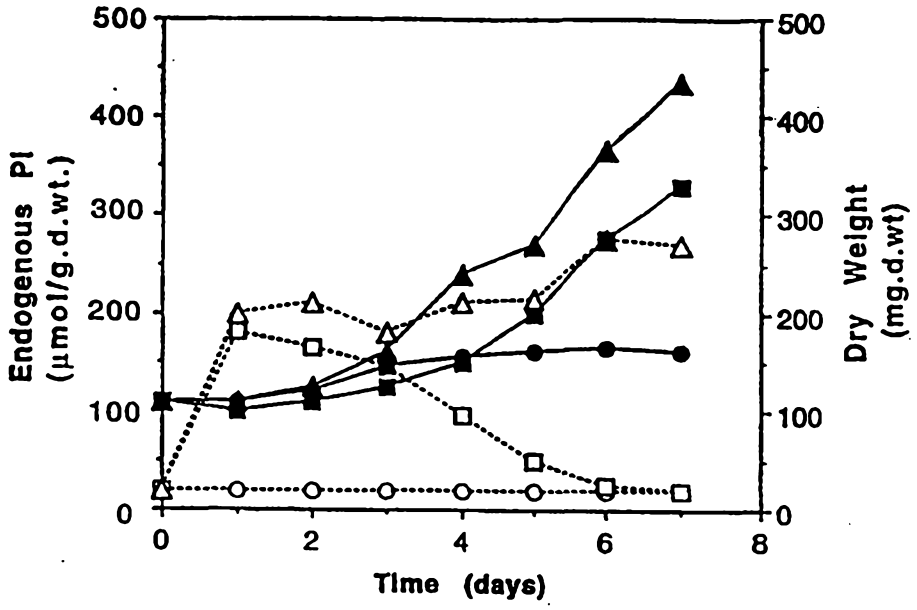

Endogenous PI
(µmol/g.d.wt.)
Dry Weight
(mg.d.wt)
Time (days)
0
100
200
300
400
500
0
2
4
6
8

FIGURE 1

FIG.2A

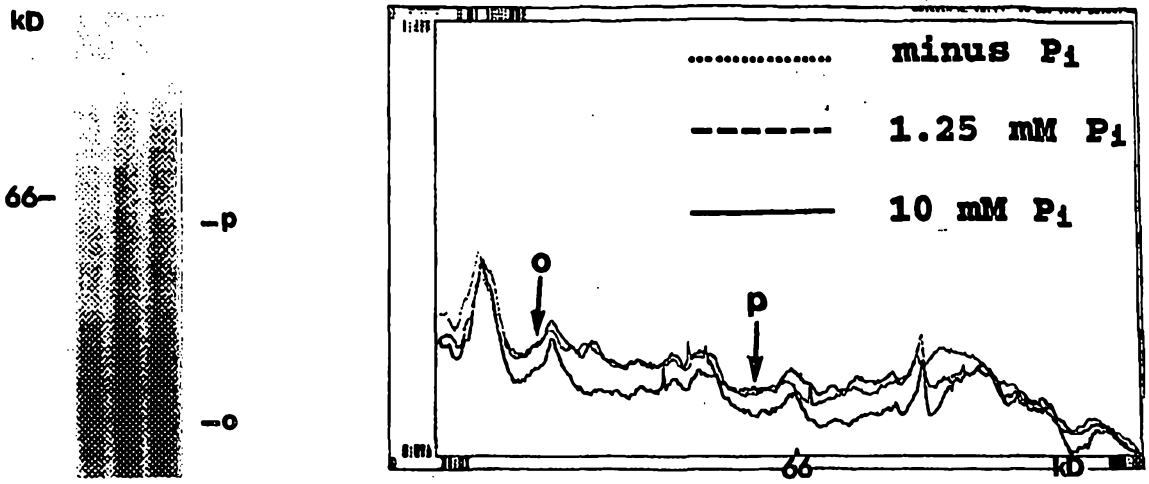


FIG.2B

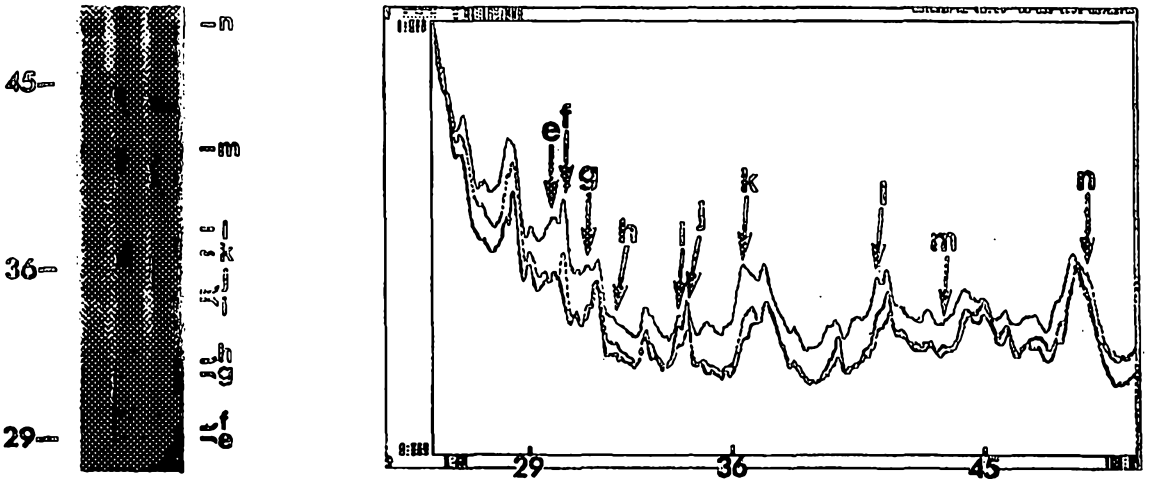
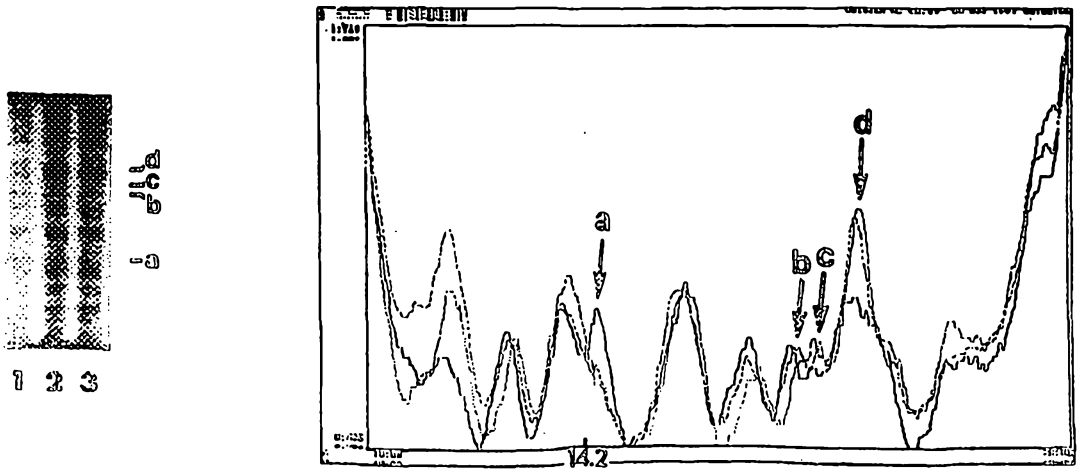


FIG.2C



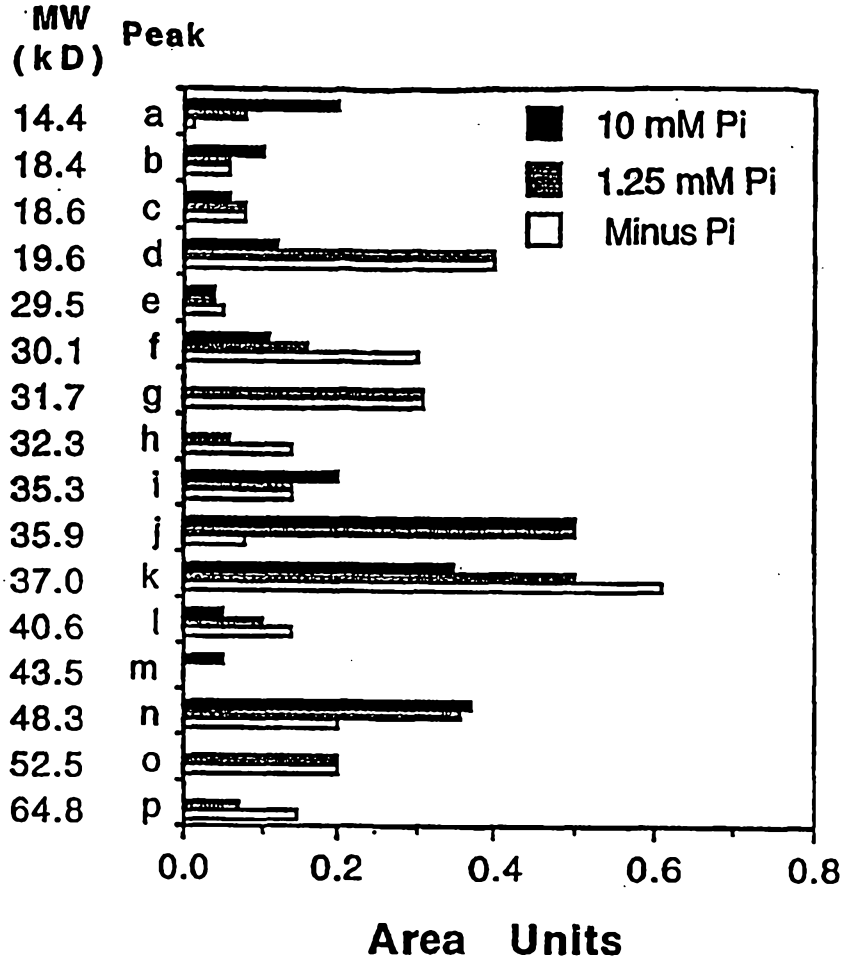
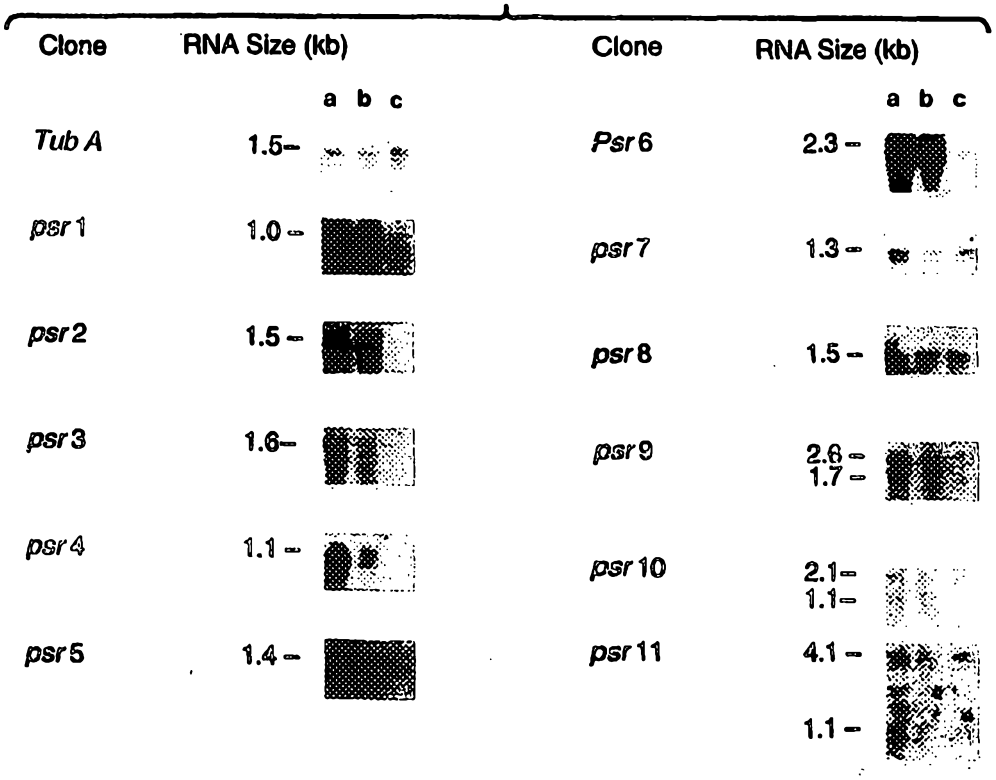

MW (kD)
Peak
14.4 a
18.4 b
18.6 c
19.6 d
29.5 e
30.1 f
31.7 g
32.3 h
35.3 i
35.9 j
37.0 k
40.6 l
43.5 m
48.3 n
52.5 o
64.8 p
10 mM Pi
1.25 mM Pi
Minus Pi
0.0
0.2
0.4
0.6
0.8
Area Units

FIGURE 3

FIG.4


Clone
RNA Size (kb)
a b c
Tub A
1.5–
psr 1
1.0 –
psr 2
1.5 –
psr 3
1.6–
psr 4
1.1 –
psr 5
1.4 –
Clone
RNA Size (kb)
a b c
Psr 6
2.3 –
psr 7
1.3 –
psr 8
1.5 –
psr 9
2.6 –
1.7 –
psr 10
2.1–
1.1–
psr 11
4.1 –
1.1 –

```
CCACGCGTCCGAGAAGATTCATCAAAAAGAAAAAAAAAAATAATAAAGGACCATTTTAGGGAAGTGAGAACAAAAACAAAA 80

GTGGTAGCTATGGAGAAGTATGAGATGGTGAAGGATTTAGGATTTGGTAATTTCGGATTGGCTCGGCTTATCCGTAATAA 160
          M  E  K  Y  E  M  V  K  D  L  G  F  G  N  F  G  L  A  R  L  I  R  N  K

GCAAACAAACGAGCTTGTGGCTGTCAAATTCATCGATCGAGGCTACAAGATAGATGAGAACGTTGCAAGAGAAATAATCA 240
  Q  T  N  E  L  V  A  V  K  F  I  D  R  G  Y  K  I  D  E  N  V  A  R  E  I  I

ATCATAGAGCTCTCAACCATCCGAATATTGTTCGGTTTAAAGAGGTTGTTTTAACTCCGACACATCTTGGAATAGTAATG 320
N  H  R  A  L  N  H  P  N  I  V  R  F  K  E  V  V  L  T  P  T  H  L  G  I  V  M

GAGTATGCAGCTGGAGGAGAACTGTTCGAGCGGATATCTAGCGTGGGTCGATTTAGCGAACGTGAGGCAAGATATTTCTT 400
 E  Y  A  A  G  G  E  L  F  E  R  I  S  S  V  G  R  F  S  E  R  E  A  R  Y  F  F

TCAACAACTCATTTGTGGAGTCCATTACTTACATGCATTGCAAATATGCCATAGAGATCTGAAATTAGAAAACACATTGC 480
  Q  Q  L  I  C  G  V  H  Y  L  H  A  L  Q  I  C  H  R  D  L  K  L  E  N  T  L

TTGATGGAAGCCCAGCACCACGTTTAAAAATTTGTGATTTTGGCTACTCAAAGTCTTCTGTTCTGCACTCCAACCCAAAA 560
L  D  G  S  P  A  P  R  L  K  I  C  Q  F  G  Y  S  K  S  S  V  L  H  S  N  P  K

TCAACGGTGGGAACTCCGGCATATATAGCACCGGAAGTTTTTTGTCGATCGGAATACGACGGAAAGTCAGTTGATGTGTG 640
 S  T  V  G  T  P  A  Y  I  A  P  E  V  F  C  R  S  E  Y  D  G  K  S  V  D  V  W

GTCTTGTGGAGTGGCCCTCTATGTTATGTTGGTAGGAGCTTATCCATTCGAAGACCCTAAAGACCCTCGCAATTTCCGAA 720
  S  C  G  V  A  L  Y  V  M  L  V  G  A  Y  P  F  E  D  P  K  D  P  R  N  F  R

AAACTGTTCAGAAAATAATGGCCGTAAACTACAAGATTCCAGGATATGTTGCACATATCCGAAGACTGCAGAAAGTTACTA 800
K  T  V  Q  K  I  M  A  V  N  Y  K  I  P  G  Y  V  H  I  S  E  D  C  R  K  L  L

TCTCGTATATTTGTTGCCAATCCGTTACATAGAAGTACGCTTAAAGAGATTAAGAGTCATGCATGGTTCCTAAAGAATTT 880
 S  R  I  F  V  A  N  P  L  H  R  S  T  L  K  E  I  K  S  H  A  W  F  L  K  N  L

GCCAAGAGAATTAAAGGAGCCAGCACAAGCAATCTATTACCAAAGGAATGTTAATCTTATTAATTTTTCTCCTCAAAGAG 960
  P  R  E  L  K  E  P  A  Q  A  I  Y  Y  Q  R  N  V  N  L  I  N  F  S  P  Q  R

TAGAGGAGATTATGAAGATAGTTGGTGAGGCAAGACCGATTCCAAACCTTTCTCGCCCGGTCGAATCGCTTGGATCAGAT 1040
V  E  E  I  M  K  I  V  G  E  A  R  P  I  P  N  L  S  R  P  V  E  S  L  G  S  D

AAAAAAGATGATGATGAAGAAGAATATTTGGATGCTAATGATGAAGAATGGTATGATGATTACGCATAGACAATAAAAAT 1120
 K  K  D  D  D  E  E  E  Y  L  D  A  N  D  E  E  W  Y  D  D  Y  A

GTATTATATGTTGTCAAATTATGAACGGTACGAACATGAACGGTACGTTCGTATTTGTAATTATCTATATGAATTTCGGT 1200

TTTTCTTTTTCATAATCACCAAATTAGTTTAAATGAAAAAAAAAAAAAAA 1250
```

FIGURE 5

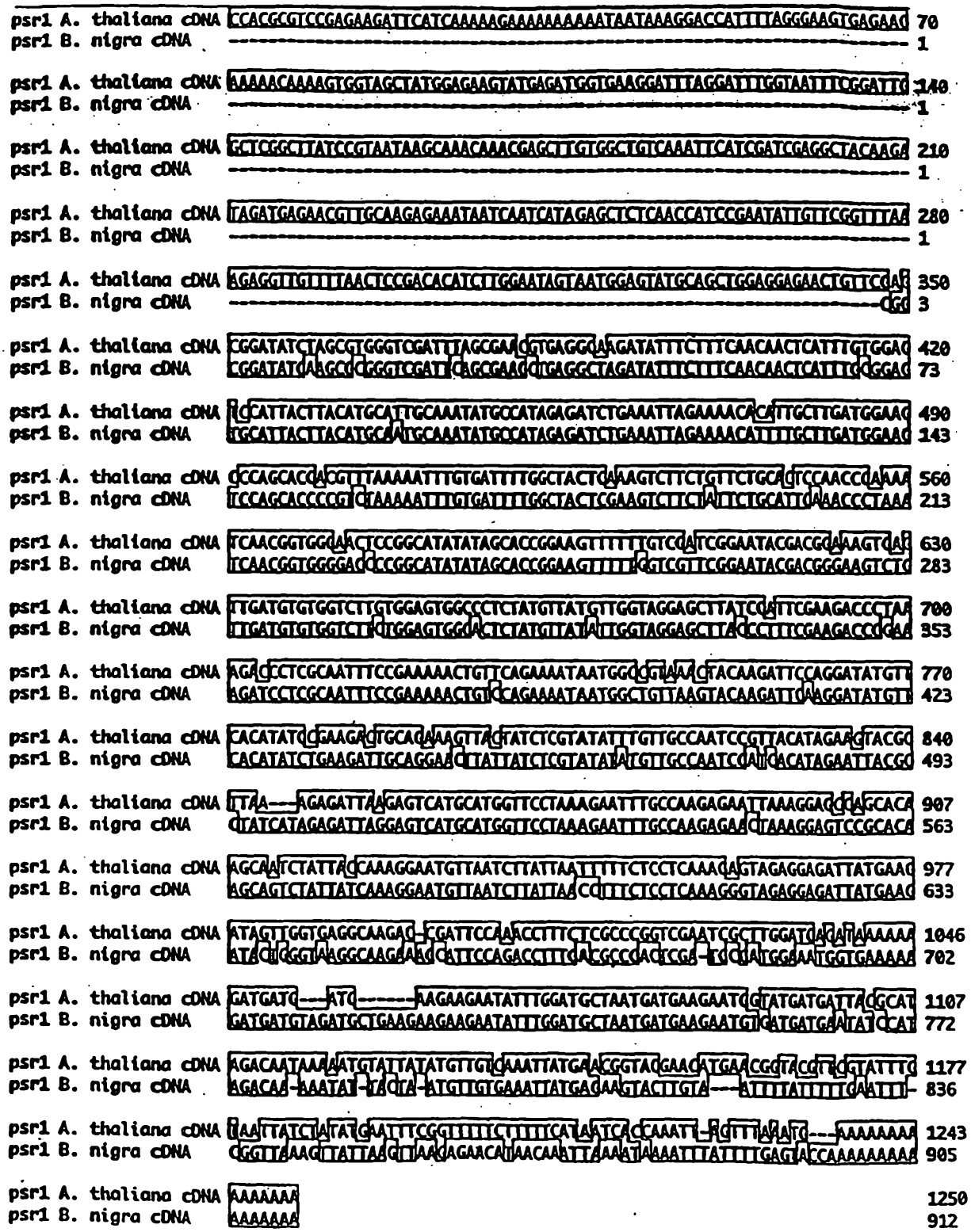


FIGURE 6

```
ccacgcgtccgagaagattcatcaaaaagaaaaaaaaaataataaaggaccattttaggg  60
aagtgagaacaaaaacaaaagtggtagctatggagaagtatgagatggtgaaggatttag  120
gatttggtaatttcggattggctcggcttatccgtaataagcaaacaaacgagcttgtgg  180
ctgtcaaattcatcgatcgaggctacaagatagatgagaacgttgcaagagaaataatca  240
atcatagagctctcaaccatccgaatattgttcggtttaaagaggttgttttaactccga  300
cacatcttggaatagtaatggagtatgcagctggaggagaactgttcgagcggatatcta  360
gcgtgggtcgatttagcgaacgtgaggcaagatatttctttcaacaactcatttgtggag  420
tccattacttacatgcattgcaaatatgccatagagatctgaaattagaaaacacattgc  480
ttgatggaagcccagcaccacgtttaaaaatttgtgattttggctactcaaagtcttctg  540
ttctgcactccaacccaaaatcaacggtgggaactccggcatatatagcaccggaagttt  600
tttgtcgatcggaatacgacggaaagtcagttgatgtgtggtcttgtggagtggccctct  660
atgttatgttggtaggagcttatccattcgaagaccctaaagaccctcgcaatttccgaa  720
aaactgttcagaaaataatggccgtaaactacaagattccaggatatgttcacatatccg  780
aagactgcagaaagttactatctcgtatatttgttgccaatccgttacatagaagtacgc  840
ttaaagagattaagagtcatgcatggttcctaaagaatttgccaagagaattaaaggagc  900
cagcacaagcaatctattaccaaaggaatgttaatcttattaatttttctcctcaaagag  960
tagaggagattatgaagatagttGGTGAGGCAAGACCGATTCCAAACCTTTCTCGCCCGG 1020
TCGAATCGCTTGGATCAGATAAAAAAGATGATGATGAAGAAGAATATTTGGATGCTAATG 1080
ATGAAGAATGGTATGATGATTACGCATAGACAATAAAAATGTATTATATGTTGTCAAATT 1140
ATGAACGGTACGAACATGAACGGTACGTTCGTATTTGTAATTATCTATATGAATTTCGGT 1200
TTTTCTTTTTCATAATCACCAAATTAGTTTAAATGAAAAAAAAAAAAAAA 1250
```

FIGURE 7

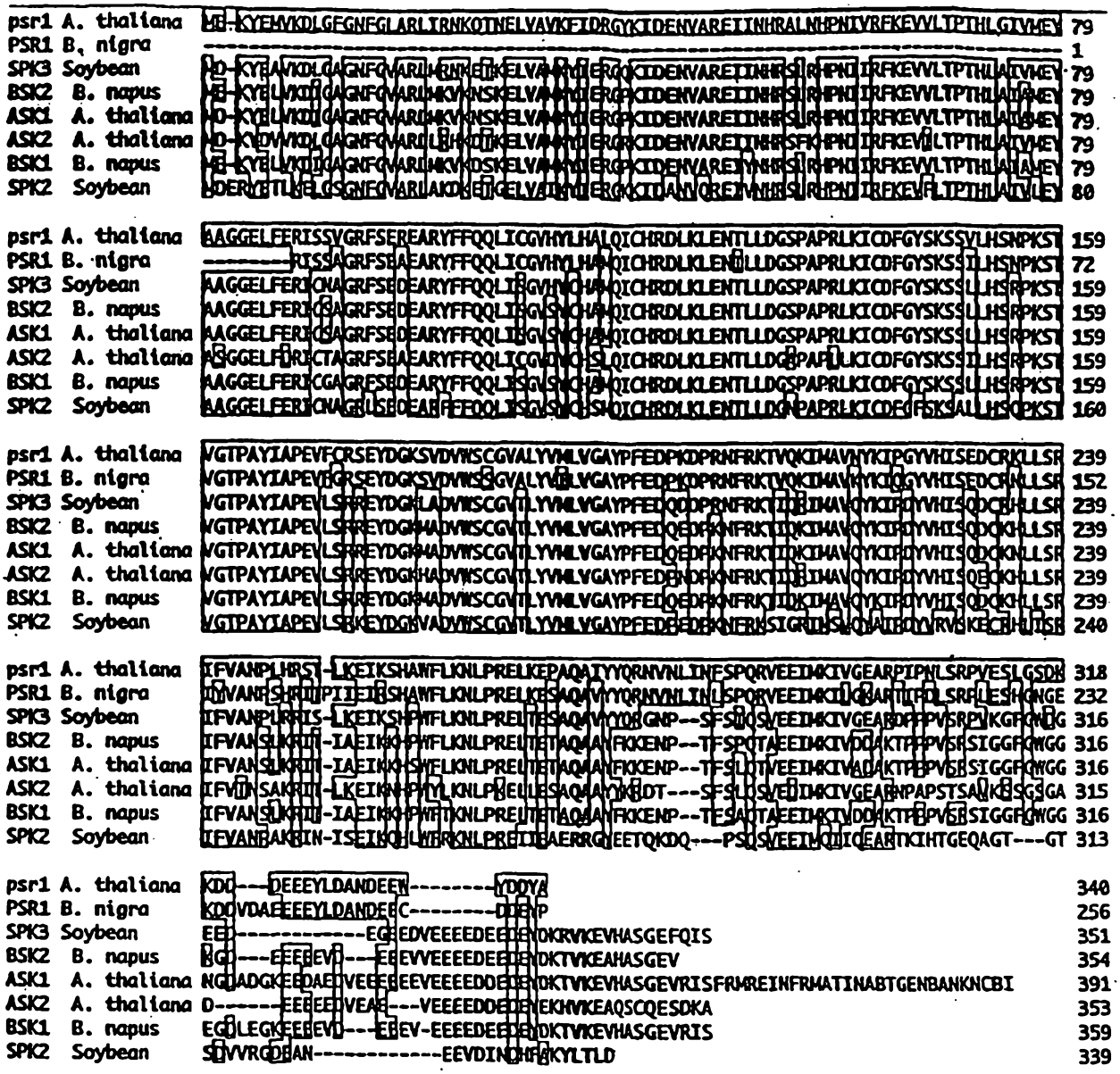

psr1 A. thaliana
PSR1 B. nigra
SPK3 Soybean
BSK2 B. napus
ASK1 A. thaliana
ASK2 A. thaliana
BSK1 B. napus
SPK2 Soybean
Decoration 'Decoration #1': Box residues that match psr1 A. thaliana exactly.

FIGURE 8

SUBSTITUTE SHEET (RULE 26)

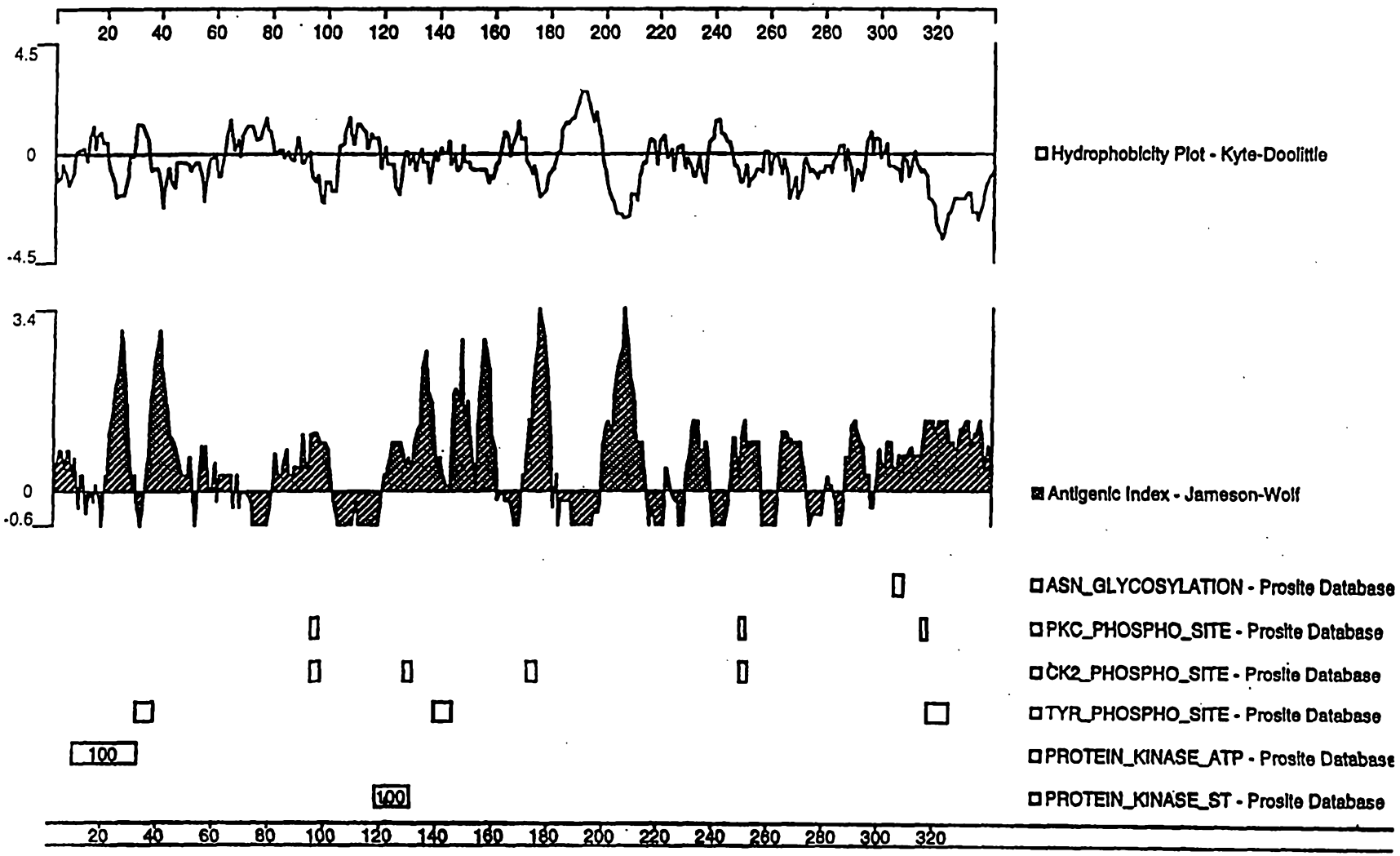


FIGURE 9

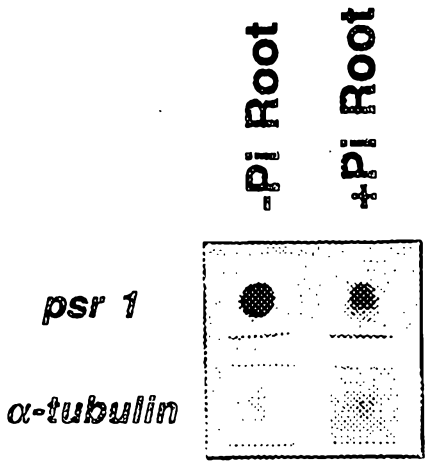
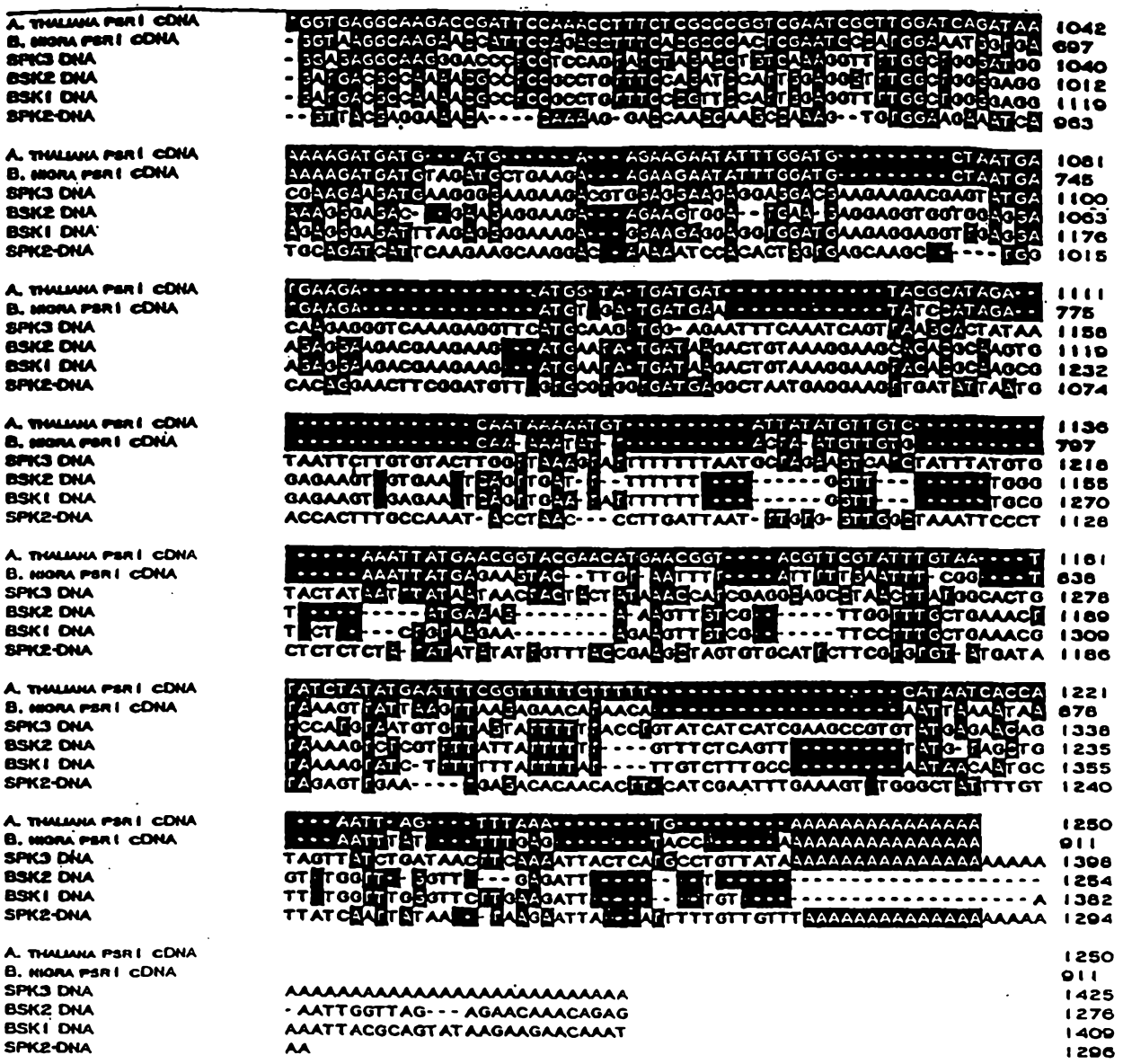


FIG.10



DECORATION 'DECORATION # 1': SHADE (WITH SOLID BLACK) RESIDUES THAT MATCH A. THALIANA PSR1 CDNA EXACTLY.

FIGURE 11

Sense Construct with Constitutive Promoter



Figure 12A

Anti-Sense Construct with Constitutive Promoter



Figure 12B

Sense Construct with Seed-Specific Promoter



Figure 12C

Anti-Sense Construct with Seed-Specific Promoter



Figure 12D

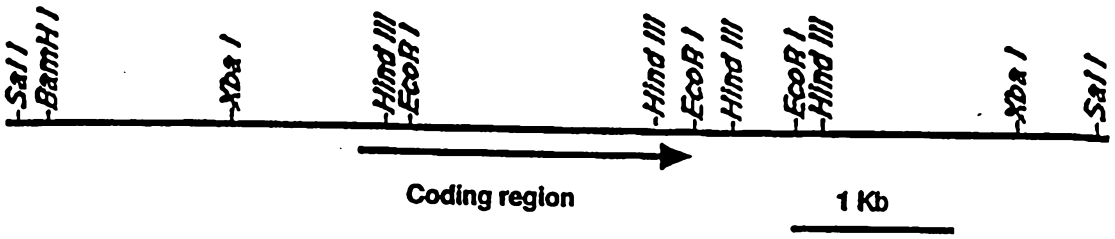

Sal I
BamH I
Xba I
Hind III
EcoR I
Hind III
EcoR I
Hind III
EcoR I
Hind III
Xba I
Sal I
Coding region
1 Kb

FIGURE 13

```
AATCATCATAAACTTGTTCTCTTCCAGAAGAAACTAAAAACAAAAATGGCATTGCAAAAGTTTCCTCTCATGGGGCTGCT 80
 •1                                                  MetAlaLeuGlnLysPheProLeuMetGlyLeuLe

TTTGCTCCTAACCATCCTCGTCTCTGTGACAACAGCGGTTGATGATCCTGTTTGCCCGGCGACTTCCAAGCTAAGCCGAG 160
uLeuLeuLeuThrIleLeuValSerValThrThrAlaValAspAspProValCysProAlaThrSerLysLeuSerArgA

CAAGTTTCCCTAATGGGTTTTTGTTTGGCACGGCTACTGCTGCGTTTCAGGTACAACAGATTTACTAAATCATAGTTCAA 240
laSerPheProAsnGlyPheLeuPheGlyThrAlaThrAlaAlaPheGln

AAAACAAAAAGTAGTGTCGTTATTGTGTTTCTATCTGAATTCAAATCCATATTTTTAAAATATGGTTTTTTTTTATGTAA 320
AAGTTGCTCAAAATATGTTATACCTATCATTTGAAATCATTTGTGCATGTGATATTCCTCAACTCATCAACTATTTTTTG 400
TTATGTCATTAGGTCGAAGGTGCAATTAATGAAACTTGTCGTGGACCGGCTCTATGGGATATCTACTGTAGAAGAAATCC 480
            ValGluGlyAlaIleAsnGluThrCysArgGlyProAlaLeuTrpAspIleTyrCysArgArgAsnPr

AGGTGAGTGTACTCGTATAATCTACATTCTCAATACAGTATGTTTAAATATTAAATTAAATTTAACTAATTAATATCAAT 560
oGlyGluCysThrG

ACATAGCCATTAATTTTCTTAATTACTTTCAAATTACAGAGAGATGTAGTGGCGACCACGCCGATGTGGCCGTTGATTTC 640
lnArgCysSerGlyAspHisAlaAspValAlaValAspPhe

TTCCATCGTTATAAGGTATATATTAATATAAATTTAAGACAAACATAACTTATATTCTCGAGATGTTATTGAAATTTTGC 720
PheHisArgTyrLys

CTTGTCTTAAAAATGTTTGTAGGAAGATATTCAGCTAATGAAGAATCTAAACACAGATGCATTCAGACTCTCAATCGCAT 800
                    GluAspIleGlnLeuMetLysAsnLeuAsnThrAspAlaPheArgLeuSerIleAlaT

GGTCAAGAATATTTCCTCGTGAGTATATGTCTGCAGATCTCTAGTTTGATTTTGTTTTAATCGAATTTGGTGAAATTAGA 880
rpSerArgIlePheProH

TTTATGTGATTGTTATATAATACTAATAACCAATGAATTTAGATGATGTATATGTGAATGTTGAAGTATCAATAACTAAT 960
GAAACAAACTGTAATGCTTTATTAGATGGGAGAAAGGAGAAGGGAGTGAGTCAAGCTGGTGTGCAATTCTACCACGAGCT 1040
                        lsGlyArgLysGluLysGlyValSerGlnAlaGlyValGlnPheTyrHisGluLe

CATCGATGAACTCCTTAAAAATGGTTATATACATATAAAATACCTAGTTCACAAAAATACAAGTAATATAGTATCCTTAT 1120
uIleAspGluLeuLeuLysAsnG

TTAACATTTCTTTCTATTTTACGTCATAAATAGGTATAGTTCCGTTTGTGACTGTTTTCCATTGGGACACTCCACAAGAT 1200
                              lyIleValProPheValThrValPheHisTrpAspThrProGlnAsp

TTGGAAGACGAATATGGCGGTTTCTTAAGCCAAAACATTGTGTATGTTTTTGTAAATTAAACAAAATACATTTAGTTAAG 1280
LeuGluAspGluTyrGlyGlyPheLeuSerGlnAsnIleVa

TTTATATGGAAATTATTAATTTGGTATCTTTTTTTTTTGCAAAAATACAGGAAAGATTTTCGAGAATATGCAGATTATGT 1360
                                                  lLysAspPheArgGluTyrAlaAspTyrVa

TTTCACTGAATACGGTGGAAAAGTGAAAAACTGGATCACTTTCAACGAGCCATGGGTCTTTGCTCACGCAGGTTACGACT 1440
lPheThrGluTyrGlyGlyLysValLysAsnTrpIleThrPheAsnGluProTrpValPheAlaHisAlaGlyTyrAspL

TAGGAAAGAAAGCACCAGGACGTTGTTCTCGCTACGTTCCAGGTTGCGAAGACCGAGAGGGACAATCTGGTAAAGAGGCT 1520
euGlyLysLysAlaProGlyArgCysSerArgTyrValProGlyCysGluAspArgGluGlyGlnSerGlyLysGluAla

TATCTAGTCAGTCACAATCTCCTCAACGCTCACGCAGAAGCTGTTGAAGTTTTCCGCCAAAAGGTAGTTTACATTAGATA 1600
TyrLeuValSerHisAsnLeuLeuAsnAlaHisAlaGluAlaValGluValPheArgGlnLys
```

FIGURE 14A

```
TAAATCATAATTAATTATCAGGTCACATTAGATATAAATCACAATTAATTATCAGGTTTCGAATCTTAAAATAGGTTGTT 1680
TTGTGTTGTGTTTATGGACATAGGTTAAAGGTGGGAAAATCGGAATCGCACATAGTCCGGCTTGGTTCGAACCACATGAT 1760
                    ValLysGlyGlyLysIleGlyIleAlaHisSerProAlaTrpPheGluProHisAsp

CTTAAAGATTCAAATGACGCTCCAACTGTTAGCCGTGTACTTGACTTTATGTTGGGATGGTAAGTATATATTATGTTAGA 1840
LeuLysAspSerAsnAspAlaProThrValSerArgValLeuAspPheMetLeuGlyTr

AAATGGTTTCTCGTATATAATTTCATAAAAATTAATGTAGTTATTAATTTTGTGTAGGCATCTGGAGCCAACTACTTCGG 1920
                                                    pHisLeuGluProThrThrSerG

GAGATTATCCACAAATCATGAAAGACCTTCTTGGTTACAGATTGCCTCAATTTACTGCTGCACAAAAAGCAAAATTGAAA 2000
lyAspTyrProGlnIleMetLysAspLeuLeuGlyTyrArgLeuProGlnPheThrAlaAlaGlnLysAlaLysLeuLys

GATTCGACCGATTTCGTAGGGCTTAACTACTATACTTCGACATTTTCAAATTATAATGAGAaGCCAGATCCGtCTAAACC 2080
AspSerThrAspPheValGlyLeuAsnTyrTyrThrSerThrPheSerAsnTyrAsnGluLysProAspProSerLysPr

AAGTTGGAAGCAAGATTCTCTTGTTTCCTGGGAACGTAAGTTTTTTTTTGTTTTCCGTACATGAAACCAAACCAAGAAAC 2160
oSerTrpLysGlnAspSerLeuValSerTrpGluP

TAAAGATAAAAGGTTTTATAATACTAATTGATATGCATACATTTTCACTTTTTATTTTTCAGCTAAGAATGTAGATCACA 2240
                                                    roLysAsnValAspHisS

GCGCCATTGGTAGCATGGTAAGCGTTTGATTAAACTAGTCCTAGTTTGATTGTTAATTTTTGTCTACGACATAACATTTT 2320
erAlaIleGlySerMet

CACCATTGCTTTATTTCAGCCTCTTACTGCTGCATTGCCCGTCTACGCTAAAGGATTTAGAAAGCTTTTAAAGTACATCA 2400
                    ProLeuThrAlaAlaLeuProValTyrAlaLysGlyPheArgLysLeuLeuLysTyrIleL

AGGACAAATACGCAAACCCGGAGATTATGATAATGGAAAATGGTAACTCGAGATTTATTGTTATATTTGTGTGTTGATTT 2480
ysAspLysTyrAlaAsnProGluIleMetIleMetGluAsnG

AATCTTAACTCTTAAGAGTAATATACTGATCATTATAATGTTGTTTTTTCTAGGATATGGAGATAAACTTGGGACCACAG 2560
                                                    lyTyrGlyAspLysLeuGlyThrThrA

ATTCGGTTGACGTTGGTACTGCTGATCATAACAGGAAATATTATCTTCAGAGGCATCTTCTGGCTATGAACGAGGCTATT 2640
spSerValAspValGlyThrAlaAspHisAsnArgLysTyrTyrLeuGlnArgHisLeuLeuAlaMetAsnGluAlaIle

TGGTAAGCCTTTTATTATTTGATAGATTGACTAACGATTCTATTGAATGGTCTAATTAAGAACATAATTGTGTTTGTGCA 2720
Cy

GCATCGATAAGGTGAGAGTTACGGGATACTTTGTATGGTCATTGTTGGATAACTTCGAATGGCAAGATGGTTACAAAAAC 2800
 sIleAspLysValArgValThrGlyTyrPheValTrpSerLeuLeuAspAsnPheGluTrpGlnAspGlyTyrLysAsn

AGATTCGGACTCTATTACGTCGATTTCAAAAATAACCTCACACGTTATGAGAAAGAATCAGCCAAGTATTACAAAGATTT 2880
ArgPheGlyLeuTyrTyrValAspPheLysAsnAsnLeuThrArgTyrGluLysGluSerAlaLysTyrTyrLysAspPh

CCTCGCTCAAGGTGTTCGTCCATCCGCACTCAAGAGGGATGAGCTTTAAGTTATATTTTGAGGATTTCGTTTTCTATTCG 2960
eLeuAlaGlnGlyValArgProSerAlaLeuLysArgAspGluLeu •

ATGTTTTTCCTATGTTTTAGTTTGTGATTGACCACGCACGATTCATGAGTCGTGGTTAATAATAATAAAAGTGTTTCGTT 3040
TTCCTTCAATCTCTAGCTTATTCGACAAGATCAAAGAAGGCTTTAGTTTTAATGACGTTATCCTTTTTGAGAATCAATAT 3120
GTGAAGTTAAATATGTTTTACCTTCCAAACTTGAAGAAAAATTGAATCATACAACCTTAACATTAAAAAATTTATGAGTT 3200
ACAAAATGATGTAAGAAAATATCATTAGTTTTAGAAAATTAAAAATACGAATTTACCTCTATACATAGAAATAGATTAGG 3280
AAATAATTTGACGTCGTCTTAACAACTAAGTACAAACATTTCAAAAACTAACAACTTAAACGTTGGCGATGAAATAAACA 3360
ATGGCAATTCATTTTCCCAAACAGTTCTAATATTTTATTTTTGAAAGTCACTAGTTTAGATTAAAAAACAGGACGACCAG 3440
CAAGAGAAGATATCTCTTTTTCACTCATTTGGCCAAGCGTATTTTCTATATACCTTAAGGAAATAAAATCCATGCATGAT 3520
GCAACCAAATAAATCCACCATGCAATACCAACATCATTCATTACTTTGGAAATTCATATTTCATTCTTAGCAAGCAAAAA 3600
AAAACAAAAAAAAAACAATCAAACGTATGTGTTAATAATGAAATCATCACATTCTATCTTACTAGTAGTCACTTATCAAAA 3680
ACGATTTATGTAAATAGTGATTTTCAAGATTTTTTTTTTAAAAGCAAAAAATTTAAAGGCCAACTCACTTTCAGACTGTT 3760
TCTTTTGTAAAATTGAGAATT 3781
```

FIGURE 14B

```
CCGGCGTTGATTTCTTCCATCGTTATAAGGAAGATATCCAACTTATGAAGAATCTAAACA 60
   G  V  D  F  F  H  R  Y  K  E  D  I  Q  L  M  K  N  L  N
CAGATGCCTTCAGAATGTCTATCGCATGGCCAAGAATATTTCCCCATGGGAGAAAGGAGA 120
T  D  A  F  R  M  S  I  A  W  P  R  I  F  P  H  G  R  K  E
AAGGGGTGAGTCAAGCTGGTGTGCAATTTTACCACGACCTTATCGACGAGCTCAAAAGAA 180
K  G  V  S  Q  A  G  V  Q  F  Y  H  D  L  I  D  E  L  K  R
ATGGTATAACTCCGTTCGTGACAGTCTTTCACTGGGACACTCCACAAGATTTAGAGGACG 240
N  G  I  T  P  F  V  T  V  F  H  W  D  T  P  Q  D  L  E  D
AATATGGTGGCTTTTTAAGTGAAAGGATTGTGAAAGATTTCCGAGAGTATGCAGATTTTG 300
E  Y  G  G  F  L  S  E  R  I  V  K  D  F  R  E  Y  A  D  F
TTTTTCAAGAATATGGTGGAAAAGTGAAACATTGGATCACTTTCAACGAGCCATGGGTTT 360
V  F  Q  E  Y  G  G  K  V  K  H  W  I  T  F  N  E  P  W  V
TCTCCCACGCTGGTTACGATGTAGGCAAGAAAGCACCAGGACGTTGCTCAAAGTACGTCA 420
F  S  H  A  G  Y  D  V  G  K  K  A  P  G  R  C  S  K  Y  V
AAGAAGAATGTCATGATGGACGATCAGGATTCGAGGCTTACCTCGTCACCCACAATCTCC 480
K  E  E  C  H  D  G  R  S  G  F  E  A  Y  L  V  T  H  N  L
TTAACTCTCACGCTGAAGCCGTTGAAGCTTTCCGACAGTGCGAAAAGTGTAAAGGTGGTA 540
L  N  S  H  A  E  A  V  E  A  F  R  Q  C  E  K  C  K  G  G
AGATTGGTATCGCACATAGTCCGGCTTGGTTTGAGCCACATGACCTTGCTGATTCACAAG 600
K  I  G  I  A  H  S  P  A  W  F  E  P  H  D  L  A  D  S  Q
ACGGTGCATCCATTGACCGTGCACTTGACTTTATTTTGGGATGGCATCTGGACACAACTA 660
D  G  A  S  I  D  R  A  L  D  F  I  L  G  W  H  L  D  T  T
TGTATGGAGATTATCCGCAGATCATGAAAGATATTGTTGGACATAGATTGCCTAAATTTA 720
M  Y  G  D  Y  P  Q  I  M  K  D  I  V  G  H  R  L  P  K  F
CCGAAGCACAGAAAGCAAAACTGAAAAACTCAGCCGATTTCGTCGGGCTCAACTATTATA 780
T  E  A  Q  K  A  K  L  K  N  S  A  D  F  V  G  L  N  Y  Y
CTTCGATGTTTTCAAACCATCTGGAGAAGCCAGATCCTGCTAAACCAAGATGGATGCAAG 840
T  S  M  F  S  N  H  L  E  K  P  D  P  A  K  P  R  W  M  Q
ATTCTCTTATTAACTGGGAAACTAAGAATGCGTACAATTACAGCATTGGTAGCAAGCCTA 900
D  S  L  I  N  W  E  T  K  N  A  Y  N  Y  S  I  G  S  K  P
TCACCGGTGCACTTCCCGTTTTTGCGAGAGGCTTTAGAAGTCTTTTGAAGTACATCAAGG 960
I  T  G  A  L  P  V  F  A  R  G  F  R  S  L  L  K  Y  I  K
ATAAGTATGGCAACCCAGAAATTATGATCATGGAAAACGGATATGGAGAAGAACTTGGGG 1020
D  K  Y  G  N  P  E  I  M  I  M  E  N  G  Y  G  E  E  L  G
CTGCAGATTCAATTGAAGTTGGTACAGCTGATCACAACAGGAAATATTATCTTCAGAGGC 1080
A  A  D  S  I  E  V  G  T  A  D  H  N  R  K  Y  Y  L  Q  R
ATCTTTTGAGCATGAATGAAGCTATTTGCATCGACAAGGTGAATGTTACCGGATACTTTG 1140
H  L  L  S  M  N  E  A  I  C  I  D  K  V  N  V  T  G  Y  F
TATGGTCCTTGTTGGATAACTTTGAGTGGCAAGATGGTTACAAGAACAGATTCGGACTCT 1200
V  W  S  L  L  D  N  F  E  W  Q  D  G  Y  K  N  R  F  G  L
ACTACATTGATTTCAAGAATAACCTCACACGATACGAGAAAGAGTCAGGCAGGTACTACA 1260
Y  Y  I  D  F  K  N  N  L  T  R  Y  E  K  E  S  G  R  Y  Y
AAGACTTCCTAAGTCAAGGTGTTCGTCCATCCATGATCAACAGAGATGAGCTTTGAGCTT 1320
K  D  F  L  S  Q  G  V  R  P  S  M  I  N  R  D  E  L  .
ACATTTGGAGGATTCAATTTCATGTTTTCTTTCTTTTGTATCCATCCGTTTGTGATTGAC 1380

CAAGATCCATGAGGTCTTGCCGGAATT 1407
```

FIGURE 15

```
AATTCCCGGGTCGACCCACGCGTCCGAGAATTACAAACAAAAATGGTTTTGCAAAAGCTT 60
                                                M  V  L  Q  K  L
CCTCTCATTGGGCTGCTTTTGCTCCTGACCATCGTCGCCTCTCCAGCAAATGCAGATGGA 120
 P  L  I  G  L  L  L  L  L  T  I  V  A  S  P  A  N  A  D  G
CCTGTTTGCCCGCCGTCGAACAAACTAAGCCGGGCAAGTTTCCCTGAAGGTTTTTTATTT 180
 P  V  C  P  P  S  N  K  L  S  R  A  S  F  P  E  G  F  L  F
GGCACGGCTACTGCGGCATACCAGGTACCAAGGTTCGATTTAATGAAACTTGTTCGTGGA 240
 G  T  A  T  A  A  Y  Q  V  P  R  F  D  L  M  K  L  V  R  G
CCAGCCTTATGGGACATCTACTGTAGAAGATATCCAGAGAGGTGCAATAACGATAACGGC 300
 P  A  L  W  D  I  Y  C  R  R  Y  P  E  R  C  N  N  D  N  G
GATGTGGCCGTTGATTTCTTCCATCGTTATAAGGAAGATATCCAACTAATGAAGAATCTA 360
 D  V  A  V  D  F  F  H  R  Y  K  E  D  I  Q  L  M  K  N  L
AACACAGACGCCTTTAGAATGTCTATCGCATGGCCAAGAATATTTCCTCATGGGAGAAAG 420
 N  T  D  A  F  R  M  S  I  A  W  P  R  I  F  P  H  G  R  K
GAGAAAGGAGTGAGTCAAGCTGGTGTGCAATTCTACCACGACCTCATCGACGAGCTCATA 480
 E  K  G  V  S  Q  A  G  V  Q  F  Y  H  D  L  I  D  E  L  I
AAAAATGGTATAACTCCATTCGTTACTGTTTTTCACTGGGACACTCCACAAGATTTAGAA 540
 K  N  G  I  T  P  F  V  T  V  F  H  W  D  T  P  Q  D  L  E
GATGAATATGGCGGCTTTTTAAGCGAAAGGATTGTGAAGGATTTCCGAGAGTATGCAGAT 600
 D  E  Y  G  G  F  L  S  E  R  I  V  K  D  F  R  E  Y  A  D
TTTGTTTTCCAAGAATACGGTGGAAAAGTGAAACATTGGATCACTTTCAATGAGCCATGG 660
 F  V  F  Q  E  Y  G  G  K  V  K  H  W  I  T  F  N  E  P  W
GTTTTCTCGCACGCTGGCTATGACGTAGGCAAAAAGGCACCTGGTCGTTCCTCTTCTTAC 720
 V  F  S  H  A  G  Y  D  V  G  K  K  A  P  G  R  S  S  S  Y
GTCAATGCTAAATGCCAAGACGGACGATCAGGATACGAGGCTTACCTTGTCACTCACAAT 780
 V  N  A  K  C  Q  D  G  R  S  G  Y  E  A  Y  L  V  T  H  N
CTCCTTATCTCTCACGCAGAAGCAGTTGAAGCTTACCGGAAATGCGAAAAGTGTAAAGGT 840
 L  L  I  S  H  A  E  A  V  E  A  Y  R  K  C  E  K  C  K  G
GGGAAGATCGGAATTGCACATAGTCCTGCTTGGTTCGAAGCACATGACCTTGCTGATTCA 900
 G  K  I  G  I  A  H  S  P  A  W  F  E  A  H  D  L  A  D  S
CAAGACGGTGCGTCCATCGACCGTGCACTTGACTTTATTTTGGGATGGCATCTAGACACA 960
 Q  D  G  A  S  I  D  R  A  L  D  F  I  L  G  W  H  L  D  T
ACTACATTTGGAGATTATCCACAGATCATGAAAGACATTGTTGGACATAGATTGCCTAAA 1020
 T  T  F  G  D  Y  P  Q  I  M  K  D  I  V  G  H  R  L  P  K
TTTACAACTGAGCAGAAAGCAAAACTGAAAGCTTCTACCGATTTCGTTGGGCTCAACTAC 1080
 F  T  T  E  Q  K  A  K  L  K  A  S  T  D  F  V  G  L  N  Y
TATACTTCAGTGTTTTCAAACCATTTGGAGAAACCTGATCCTTCAAAACCAAGATGGATG 1140
 Y  T  S  V  F  S  N  H  L  E  K  P  D  P  S  K  P  R  W  M
CAAGATTCTCTTATTACATGGGAGTCTAAGAATGCGCAAAATTACGCCATTGGTAGCAAG 1200
 Q  D  S  L  I  T  W  E  S  K  N  A  Q  N  Y  A  I  G  S  K
CCTTTGACCGCTGCATTGAACGTTTACTCGAGAGGTTTTAGAAGTCTTTTGAAGTACATT 1260
 P  L  T  A  A  L  N  V  Y  S  R  G  F  R  S  L  L  K  Y  I
AAGGACAAATACGCAAATCCGGAAATTATGATCATGGAAAACGGATATGGAGAAGAACTA 1320
 K  D  K  Y  A  N  P  E  I  M  I  M  E  N  G  Y  G  E  E  L
GGGGCCTCAGATTCTGTTGCTGTTGGTACCGCTGATCATAACAGGAAATATTATCTTCAG 1380
 G  A  S  D  S  V  A  V  G  T  A  D  H  N  R  K  Y  Y  L  Q
AGGCATCTTTTGAGTATGCAAGAAGCTGTTTGCATCGACAAAGTGAATGTTACAGGATAC 1440
 R  H  L  L  S  M  Q  E  A  V  C  I  D  K  V  N  V  T  G  Y
TTTGTATGGTCATTGTTGGATAACTTCGAGTGGCAAGATGGTTACAAAAACAGATTTGGA 1500
 F  V  W  S  L  L  D  N  F  E  W  Q  D  G  Y  K  N  R  F  G
CTCTACTACGTTGATTTCAAAAATAACCTCACACGTTACGAGAAAGAATCCGGCAAGTAT 1560
 L  Y  Y  V  D  F  K  N  N  L  T  R  Y  E  K  E  S  G  K  Y
TACAAGGATTTCCTCAGTCAAGGTGTTCGTCCATCCGCGCTCAAGAAGGATGAGCTTTAA 1620
 Y  K  D  F  L  S  Q  G  V  R  P  S  A  L  K  K  D  E  L  .
GCTATTTCTGTTTCAATGTGTTTTTCCTATGTTTTACTTTGTGAGTGACCAAGATTCATG 1680

AGGTCTTGGTTCTAATAAAAAGAGTTTATTTTTCTTCTCATTTTTCATTGTCTACATGAT 1740

TTGCCAGATCTATAAGGCTCTGGTTATAATAAAATGATCCTTTGTGCCTAAAAAAAAAAA 1800

AAAAAAAAAAAAAAAAAAAAAAAAGGGC 1829
```

FIGURE 16

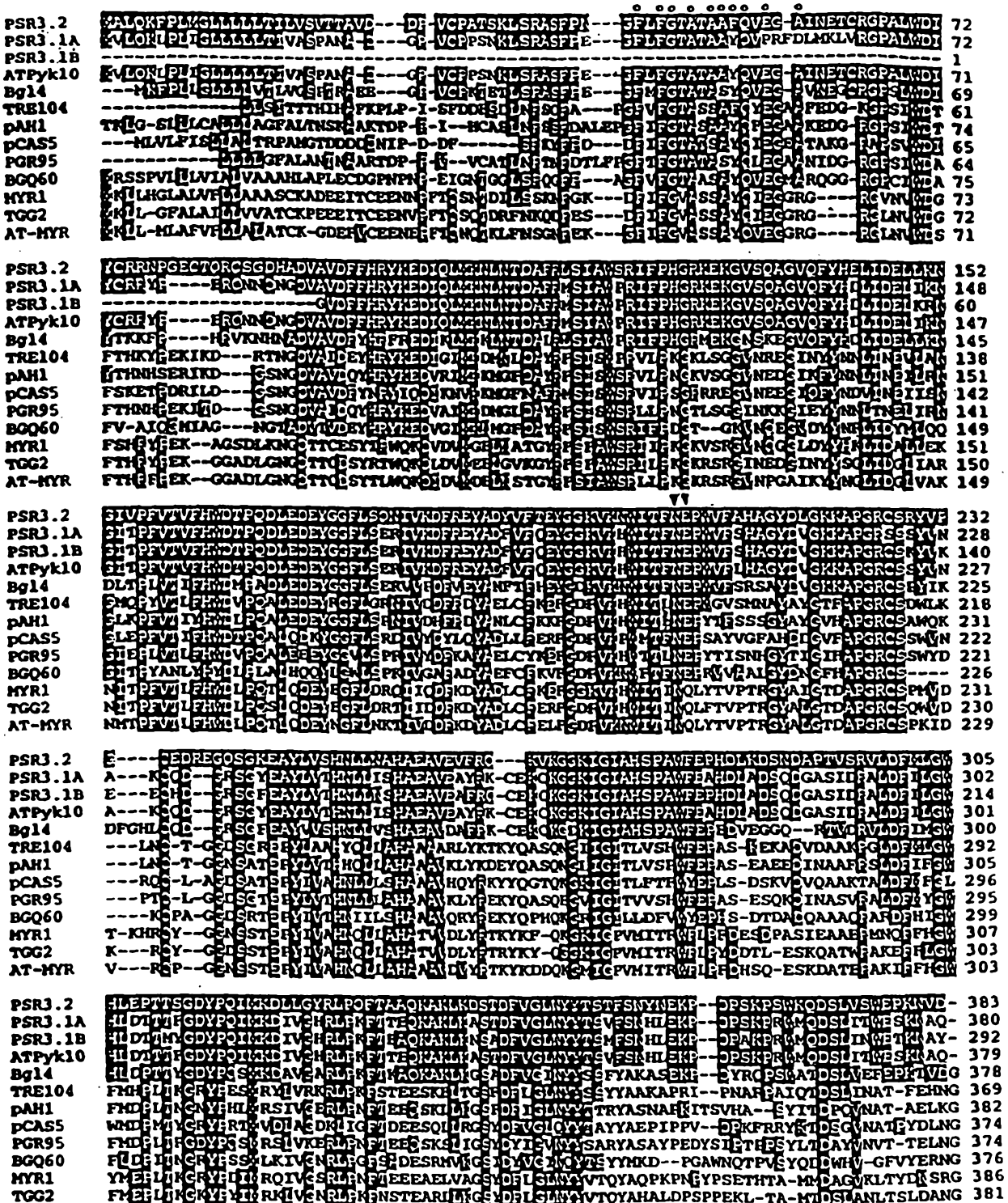


FIGURE 17A

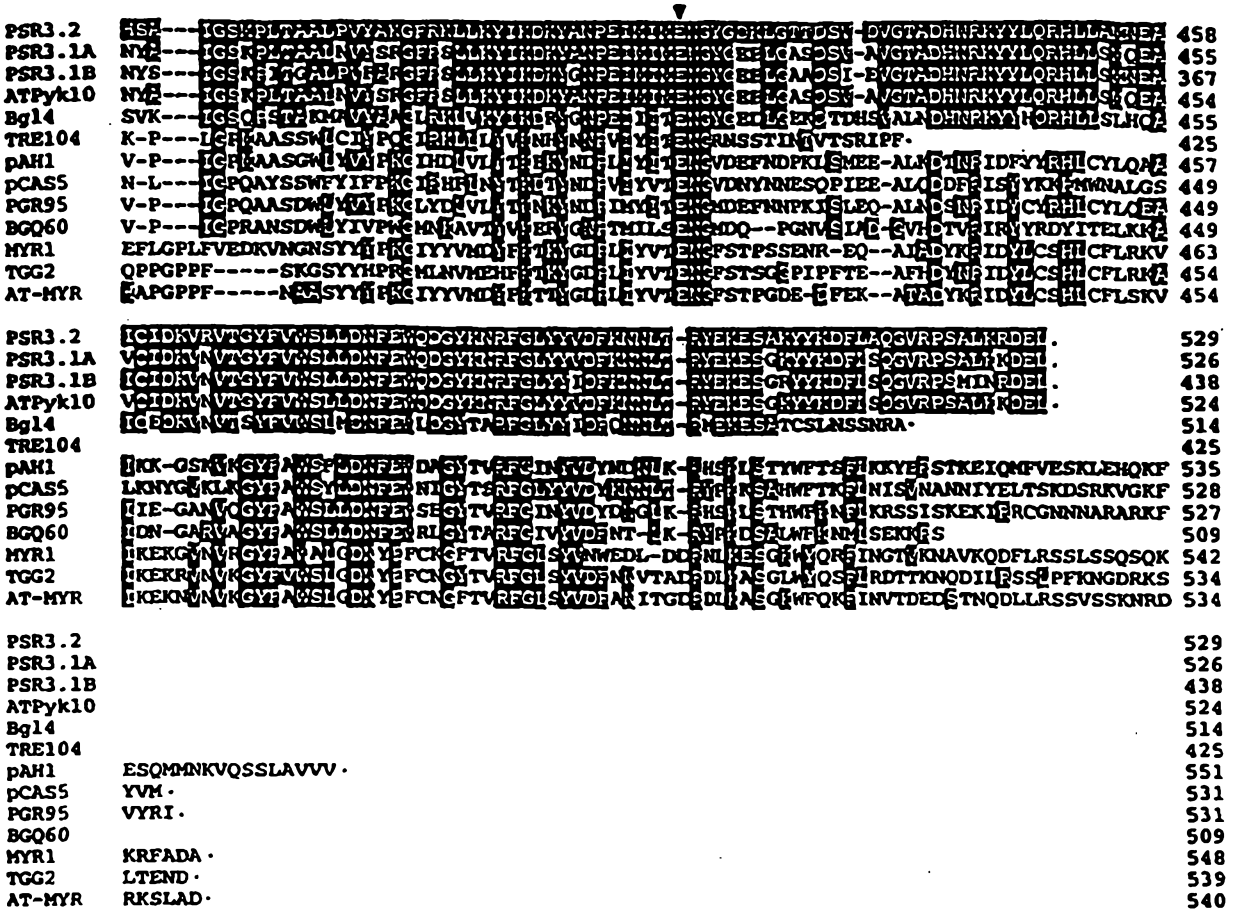

PSR3.2
PSR3.1A
PSR3.1B
ATPyk10
Bg14
TRE104
pAH1
pCAS5
PGR95
BGQ60
MYR1
TGG2
AT-MYR

FIGURE 17B

Name: *psr2*

Insert size: 950 bp

mRNA size: 1.5 kb

Estimated protein size: 42 kD

T3)

AATTCCXXGGCAATGATCATGAATAAXCCTGTGAACTTCTTTGTGGTTGA 50
TGCGTTCACTGAGTCAXCTTTCAAAGGGAACCCAGCAGCAGTGTGCATTC 100
TTGAAGAGGATTATGAGAGAGACXACGCATGGCTTCAGTCTCTTXCCGCA 150
GAGTTTAACGTTTCTGAAACTTGTTTTGTGTXTCCCATTACTGGTCACGA 200
TGGTCXCXCCTCCGGTGGTTTACXCCTTCACTCGAGATGGATCTTTGTGG 250
TCATGGAACATTGGCATCTGCCTTATAGCCTCTTCCTCAAACGGXXGGTT 300
GATTCAGACAAGGCACGAGTTTXTAACACAATCAGGTATTCTTACAGGCC 350

T7)

AATTCCGGAGTCACAGCTTTTCCAGTGAGAAAAACTCTCTGCTCCTCCTT 50
ATCGTAACGGAACCTTCgACTGTTCCACTCCTACACGAAGCCGAGTAAG 100
CTRcGAAATCACACTTGTTCATCTTGAGGCTCCAGTAATGTGCTAATGGA 150
CAATGTGCACTTCCACATACAGGGTCCTCATCCACTCCTAATCTGGGGGA 200
AAAGAACCGACTGCAGAAATCATACGCAGATCCTTCAGGAGCAGCAGCTG 250
TAACAATGATCAACTTTCGGGACATTTTGAAATTCA 286

FIGURE 18

Name: *psr4* 21/30

Clone No.: C53
Insert size: 0.8kb
mRNA size: 1.1kb
Estimated protein size: 32kD

T3)

```
AATTCCGGTTTTTTTTTTAATACATAAAAATGAAATTATTTTATTTCATT 50
TCATTGCAATACATTGCAGCATTTCATTTCATTACAATACATTXCAGCAT 100
TATGCATGCATGATGCATGACTAAGACCTGGTAACAGAAGTTACGACCAC 150
AATACTTTAAAACATAGAGACATACGCCTTATTATACCTTATTCATATTT 200
TTAGACCTTTTTCATGCATACACAAGCAAACATATTAGGTCATGACACAA 250
AACATAAAACAGCAGAACGGAAACACACCGTAACCAAACCAAGAAGGGAT 300
ACTTAATTTGTTTGAAATCGGAGTATTAAGGGTGACAGTGAGAGGGGTGA 350
CCAGTTCTCTGAATACCTTCCAGTTCAGCCGACGG 385
```

T7)

```
AATTCCGGTTTTTTTTTTATACTCTCTACTCTTTGATATCCTAACAACAT 50
GGCAATGCTCGTCAGAAACAAAAACATAACCTTCTCACTGGTCTTGATAT 100
GTCTAATTGTGGTGTCTCCAGTGGCTAAGGCTCAACTTGATGGGCTTCTT 150
GGCAGGGATCCCAAAATCGTCAATATACAAGGGCGTATGATGTGCTCTAT 200
CGATGGTAATCCGAATTCCACTCCTCCAGTTGGTTCTCCCGTTATGCTTC 250
AGTGTGGTGGAAAAAATGTTGCAACTACGAATACGGTCGTTGGTGGAGGA 300
TTCTCGTCCTTCCGACACAGTGTCTACTATXCTTTCAGAACATCATCAAC 350
GA 352
```

FIGURE 19

Name: *psr5*

Clone No.: c28
Insert size: 1.9 kb
mRNA size: 1.4 kb
Estimated protein size: 40 kD

T3)

```
AATTCCGGAT CAAGAAAAGG TCTGAATCCA ATATCAAATA GAACATTTGC  50
AGAGATGACC ATGTATGTAG TCATGATTCA AAGGACAAAA CAATTGATTC  100
CAGACAAAAC AAAACGATGA TCACAACGTT ACGGCGAGGT GGCGCTGACG  150
TGACGGTGGC GTCCGTCGAG GCTCAGGTTG GCGTTGATGC TTGTCATGGT  200
ATTAAGATGG TCGCTGATAC TCTCCTCTCT GATATTACCG ATTCTGTTTT  250
CGACCTTATT GTGCTCCCCG GAXCTTCCCG XXXCGAGACT CTTAAAAACT  300
GTAAACTTTG GAGAATATGG TAAAGAAACA AGCACGAGAT GGACGACTTA  350
AAGACAAAAC  351
```

T7)

```
GATCGACGGT ATCGATAAGC TTGATATCGA ATXCCGGGCT CAACAGGAGA  50
TCATTTGCAC AGACCGTAGA GAATTTGTTC GCTCTATCTT TCTTGTCCAA  100
AGACGGACGA GTAGAGATCA TTGTTGATAA GAATGGCTCA CATTTTGCCT  150
TGCCGAGAAA CGCCACCAGC TGCGAACCTG GTGGCGTCAG GGGAAGTCAC  200
TTACAACCAC TTTGTGATTT AGATTCGATT CAAGACTGGA GATGATGTCT  250
GAAATGGTGC CGATGGGGGA AGAGCTAAGT TACCCACACA GAGAAA  296
```

FIGURE 20

```
GAATTCCGGGAATGACGTTGATGGTCATGGTGCTTTGCTCCGTAGCCTCTGGTCTCTCTT 60
   I  P  G  M  T  L  M  V  M  V  L  C  S  V  A  S  G  L  S
TTGGAGACAAACCAAAATCCGTGATGACCACGCTATGTTTCTTTAGGTTTTGGCTAGGGT 120
F  G  D  K  P  K  S  V  M  T  T  L  C  F  F  R  F  W  L  G
TTGGGATCGGTGGTGACTATCCTCTGTCCGCAACAATCATGTCTGAGTACGCTAACAAGA 180
F  G  I  G  G  D  Y  P  L  S  A  T  I  M  S  E  Y  A  N  K
AAACTCGTGGAGCGTTCGTTTCTGCGGTTTTCGCTATGCAAGGGTTTGGTATCATGGCTG 240
K  T  R  G  A  F  V  S  A  V  F  A  M  Q  G  F  G  I  M  A
GTGGCATCTTCGCTATAATCATCTCCTCTGCTTTTGAAGCTAAGTTCCCTGCACCGGCCT 300
G  G  I  F  A  I  I  I  S  S  A  F  E  A  K  F  P  A  P  A
ATGCGGAAGATGCCTTGGCATCCACGGTTCCTCAAGCGGATTTCGTATGGCGTATAATCT 360
Y  A  E  D  A  L  A  S  T  V  P  Q  A  D  F  V  W  R  I  I
TGATGGTTGGTGCCATCCCTGCAGCGATGACGTATTACTCAAGGTCCAAGATGCCTGAAA 420
L  M  V  G  A  I  P  A  A  M  T  Y  Y  S  R  S  K  M  P  E
CCGCAAGATACACAGCTCTGGTTGCTAAAGATGCAAAGCAGGCTGCTTCAGACATGTCTA 480
T  A  R  Y  T  A  L  V  A  K  D  A  K  Q  A  A  S  D  M  S
GGGTTCTGCAAGTGGAGATAGAGGCAGAACAGGAGAAAGTTGAAGAGATCTCAAGTAATA 540
R  V  L  Q  V  E  I  E  A  E  Q  E  K  V  E  E  I  S  S  N
AGTCCAAAGCCTTTTCCTTGTTCTCCAAGGAGTTCATGAAACGCCATGGGCTTCATTTGC 600
K  S  K  A  F  S  L  F  S  K  E  F  M  K  R  H  G  L  H  L
TTGGGACTACATCCACTTGGTTCCTTCTCGATATCGCTTTCTACAGTCAAAACCTTTTCC 660
L  G  T  T  S  T  W  F  L  L  D  I  A  F  Y  S  Q  N  L  F
AGAAGGATATTTTCAGCGCAATTGGGTGGATTCCTCCAGCTCAAACCATGAACGCGATTC 720
Q  K  D  I  F  S  A  I  G  W  I  P  P  A  Q  T  M  N  A  I
AAGAAGTTTTCAAGATCGCACGTGCGCAAACTCTCATCGCCTTGTGTAGCACGGTACCTG 780
Q  E  V  F  K  I  A  R  A  Q  T  L  I  A  L  C  S  T  V  P
GTTACTGGTTCACAGTTGCGTTTATAGATGTCATTGGAAGGTTTGCGATTCAGATGATGG 840
G  Y  W  F  T  V  A  F  I  D  V  I  G  R  F  A  I  Q  M  M
GATTCTTCTTCATGACGGTCTTTATGTTTGCTTTGGCTATTCCGTACAACCACTGGACTC 900
G  F  F  F  M  T  V  F  M  F  A  L  A  I  P  Y  N  H  W  T
ACACGAGAAACAACTTAAATTGCTGGAAACTGAAAAAAAGACTCAACGACTGAAAAAAAA 960
H  T  R  N  N  L  N  C  W  K  L  K  K  R  L  N  D  .
AAAAAA 966
```

FIGURE 21

Name: *psr7*

Clone No.: c112
Insert size: ? kb
mRNA size: 1.5 kb
Estimated protein size: 36 kD

T3)

```
CTCCACCGCG GTGGCnnCGC TCTAGAACTA GTGGATCCCC CGGGCTGCAG   50
GTTTTTTTTT TTTTTTTTTT TGATTATATA TGATTTTATT ATTGAAACAA  100
AAAGCTCAGA GGATAGATTG TATCAGTCTT AAATAGAACT ACACATCACT  150
ATTTAAATAA ACnCCnGCGA CTTAACTGAA GAAnCGCCGG AAAATAAGAA  200
GAACAAAACA GGGAAGGAAG GTAGATGACT AGGTTAGCCT AATAAACAAA  250
ACTGCGGGCG  257
```

T7)

```
AATTCCGTTG AATCACGCCG CTTTGCTGAA GGATGAGCTC GACATTGTGA   50
TTCCTACCAT CCGTAACCTC GAGTTCCTCG AGATGTTGGA GGCCTCTTCT  100
CAGCTACATC TCATCATCGT CAGGACGGAG ATCTTAAGAC ATCGTGTTCT  150
GAAGGGTCGA TTACXAGCTC TACAACAGGA ACGACATTAC GATCTGGCCC  200
AAAGCTTCCT GCATTTCCTT CAAGGACTCT GCTTGT  236
```

FIGURE 22

Name: ***psr8***

Clone No.: c71
Insert size: 0.4 k
mRNA size: 1.5 kb
Estimated protein size: 42 kD

T3)

```
CCTCCXCAAT XXCXXCGCTC TAGAACTAGT XXXTCCCCCG  40
GGCTGCAGGA ATTCCGGATG TGATXGTCAT CTAGGAAGAA  80
CGTAAATATG TGAAGAGTTT CGACAACAAC AGTCXXXGCA 120
TATAGTAATC AAAAACTCTT TTTCAATCTA CCAAACAAAA 160
CAAAATGAxX XCATAGGGAC XAAXCCCAGC CCATAAAGGA 200
TCTCCACCTA GAGGCCAGAA AATTACT  227
```

FIGURE 23

Name: *psr9*

Clone No.: c100
Insert size: 1.6 kb
mRNA size: 2.6 and 1.7 kb
Estimated protein size: 72 and 47 kD

T3)

```
AATTCCGGCTTGGAGAAGAAGCACATCTGGACCCAGCTCTTCTTCGGCTC 50
TTAACCACTAGTGACTTCGCGTTTCTCGTCTCCACAAGATCATTCATGTA 100
CTGAACAACGGCCTGAGGGCCTTTTCGGCTATATCACGAGGAGGAACTTG 150
TAGCGGATTCTCCTGAGCACGAAACACACGCAGCTTCGTAAGCATTCTAA 200
AAGACTCAGGAAGAACACGGATCTGGTTATTGCTGATATCAAGCTCCTCG 250
AGCATCTCAAGGTTCCCTAACCGACCTCGGCAACGATATCATATCAGCAA 300
AGTTGTTCCCACAGCTTCACAAGCGAC 327
```

T7)

```
AATTCGCTGCAGCTGGATAAGCGTCTGGACACGACGGCAGCAAGTGGTGG 50
AAGAGATCGATGAGAATCCACAGATCTCTACCGCCGAGACCAGGCTCGAC 100
GAGGTCGAAGCCGCGAGGTCTCTGATACAGAACgtgGAGAAGGAAGACCA 150
ATCCTGGCTAGAAGccATTgCTAACcAGAGGAAACCCTCCGAGGTTCCCG 200
ACGAGCTATTCGCCGTGCTGCAGGAGATGAAGAGAGGTTCGTTCGGTTTC 250
GTAGCAAAGAGCAGATAAGAGAAGCCGCCAAGCTTCTCGATCTGGAAACG 300
GTCACTCTCACTTCGACGATTATCAGAGAGCTTCTGATTGCATTGCTTCT 350
CTTCTCGACGG 361
```

FIGURE 24

Name: *psr10* 27/30

Clone No.: c70
Insert size: 0.4 kb
mRNA size: 2.1 and 0.8 kb
Estimated protein size: 59 and 23 kD

T3)

```
ACCGCGGTGG CGCCGCTCTA GAACTAGTGG ATCCCCCGGG CTGCAGGTTT  50
TTTTTTTGTA TATAAGAAAA TGGTTGATTT ATATACATGA TTTACAACTA 100
GTAATTCCCC AAAAAATCAG CAGAGAGAAA AAGATATCGG AACAGGACTC 150
GATTGTTGAC CATGACCACT CGTCTCCATG GTCTTCATGG CGTCTCAGcC 200
CCCCCTAGCA CTTTAGTTTC TCTTGAA  227
```

T7)

```
TCAGCTCGCC GCCAAGATCC TAGGAAAGCT TTTAGGGTTT TCGTGTCAGA  50
CTCTTCGTTT TTTTTTTTTT GGTTCACGAC CATCTTAAGA TCTTATTCTC 100
TTCTTTTCTT TGCCTTGGGC GAGCTTGATT ACGACGCATG TTGCGTTTTG 150
CTGTCTAAGT TTTTGATTCT CTTCAAAGTA AAAATAGTGA TACATTATAT 200
CTGGAAAGAA AACTAGTGAT TCCGATCT  228
```

FIGURE 25

Name: ***psr11***

Clone No.: C59
Insert size: 0.8 kb
mRNA size: 4.1 and 1.1 kb
Estimated protein size: 115 and 32 kD

T3)

```
AATTCCGGTT TTTTTCAATG AAATAACTGT ATGGTTAATA ACTTGATATA  50
GCAATATAGA GTCTAGACCA ACGTACAGTT TACAAAGAAA GAGAAGATGA 100
AAAGCAGTAC TTTGGGTTTC AGAACAAGGC TTTTATTGAA GGACCCTTTA 150
TTGAACCAAT ACCATAACCT ATAGGATCAA AGGCCTAAAA GAGAGGTAAG 200
CTTGTAGGGA AACGAACTTG CATCCACTGC TTCTCATACG TAACCAATGG 250
CTCCAGCCTA ACTCTCAGCA ACCAGTTCAT TGCAAGTTCT AGAGCG  296
```

T7)

```
AATTCCGGGG CAAGTCGACC TCCAGTTATT TCATTTCAAC AAATGTCTTT  50
CAACTCAGGC CACATTGAAC GAAGATTTAT GGAGGTACCA CTTGGGGCAA 100
CATGGGCTGA GGCTACGATG CGAACTTCGG GGTTTGATAC TACACGGAGA 150
TTTTATATTG ATGCGCTTCA GATTTGCCCG TTGAGAAGGC CTATCAAGTG 200
GGAGAACGCA ACAACATTTG CATCTCCATC TGCTAAAAGC TTTGCGTTTC 250
AGTGGTTAGT GGTCAAACGA TGGAACTAGC TCTAGCTCAA TCTGGGTCAG 300
TGGCTGGAGT GCAACCAAC  319
```

FIGURE 26

FIG.27A

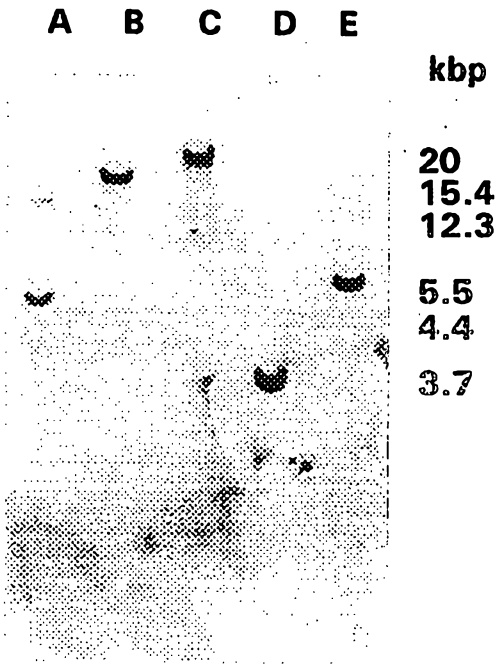
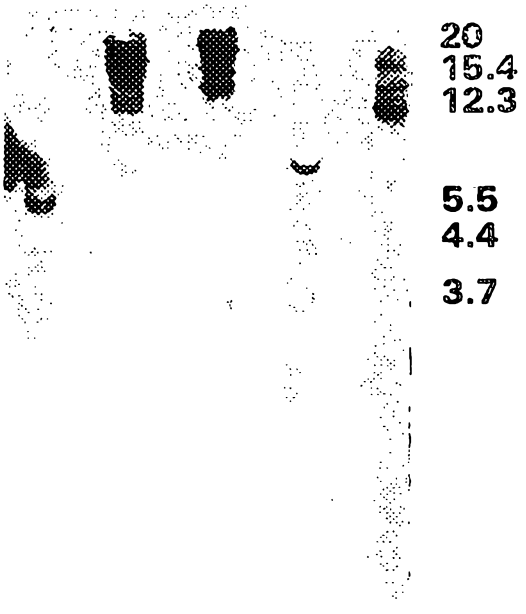


FIG.27B



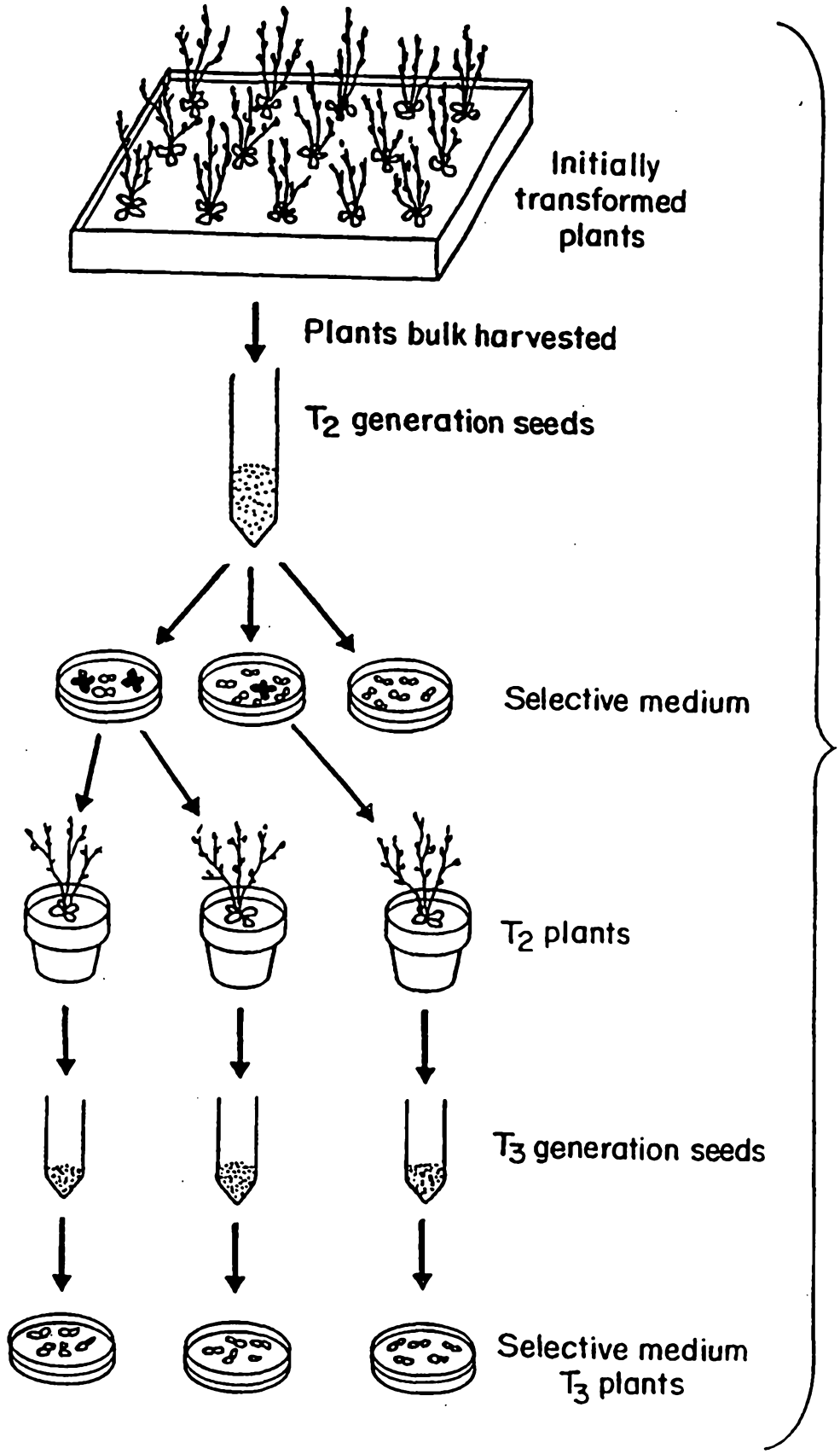

Initially transformed plants
Plants bulk harvested
T_2 generation seeds
Selective medium
T_2 plants
T_3 generation seeds
Selective medium T_3 plants

FIGURE 28